UNIVERSITY OF ALBERTA

Actions of GnRH and dopamine on identified goldfish gonadotrophs: ionic currents and intracellular calcium

BY

FREDRICK FRANS VAN GOOR ©

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Actions of GnRH and Dopamine on Identified Goldfish Gonadotrophs: Ionic Currents and Intracellular Calcium submitted by Fredrick Frans Van Goor in partial fulfillment of the requirements for the degree of Doctoral of Philosophy.

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THIS THESIS IS DEDICATED TO MY MOTHER AND FATHER
IN THANKS FOR THEIR ENDLESS SUPPORT

ABSTRACT

Studies of goldfish pituitary cell populations have suggested that gonadotropin-releasing hormone (GnRH) stimulates maturational gonadotropin (GTH-II) secretion by activating protein kinase C (PKC), increase in Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCC) and elevations of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). It has also been proposed that dopamine inhibits GTH-II secretion by reducing Ca^{2+} influx through VDCC and thus reducing $[Ca^{2+}]_i$ elevation. To directly test these hypotheses, electrophysiological and fura-II Ca^{2+} imaging studies were performed on individual goldfish gonadotrophs.

To facilitate these studies, a reliable method to identify goldfish gonadotrophs based on their unique cellular morphology when viewed under Nomarski differential interference-contrast microscopy was developed. This novel technique for cell identification was shown to be applicable to somatotrophs and lactotrophs.

Patch-clamp recordings indicated that goldfish gonadotrophs are electrically excitable and exhibit voltage-dependent Na^+ currents, VDCC, as well as multiple K^+ current sub-types. Unlike mammalian gonadotrophs, apamin-sensitive K^+ currents were not observed in goldfish gonadotrophs. Activation of dopamine D_2 receptors reduced current amplitude through VDCC, but did not alter voltage-dependent Na^+ or K^+ currents. Thus, VDCC are an important site for the regulation of GTH-II secretion by dopamine.

GnRH and PKC activators stimulated a rapid, localized increase in $[Ca^{2+}]_i$, but did not directly affect VDCC. Since this response was relatively unaffected by the inhibition of Ca^{2+} influx, these results suggest that the acute $[Ca^{2+}]_i$ increase is largely mediated by mobilization of intracellular Ca^{2+}. Although VDCC do

not appear to directly participate in acute GnRH action, their activity may regulate the concentration of Ca^{2+} in intracellular Ca^{2+} stores.

Amiloride-sensitive Na^{+}/H^{+} antiports also participated in agonist-stimulated GTH-II secretion. Activation of these antiports may be important to counteract cytoplasmic acidification resulting from agonist-induced increases in $[Ca^{2+}]_i$.

In conclusion, these results support the hypothesis that VDCC and $[Ca^{2+}]_i$ are targets for neuroendocrine regulation in gonadotrophs. Furthermore, they identify the importance of intracellular Ca^{2+} release and cytoplasmic pH regulation.

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ABBREVIATIONS.

1, 2-Dioctanoyl-*sn*-glycerol	DiC8
4-Aminopyridine	4-AP
5-(N, N-dimethyl)-amiloride	DMA
Analysis of variance	ANOVA
Bovine serum albumin	BSA
Cell capacitance	C_m
Chicken GnRH-II	cGnRH-II
Cyclic adenosine-3′,5′-monophosphate	cAMP
Cytosolic free Ca^{2+} concentration	$[Ca^{2+}]_i$
Diacylglycerol	DAG
Dimethyl sulfoxide	DMSO
Fisher's least significance difference test	LSD
Follicle-stimulating hormone	FSH
Fura-II acetoxymethyl ester	Fura-II AM
Gonadotropin-releasing hormone	GnRH
Gonadotropin	GTH
Growth hormone	GH
guanine nucleotide binding protein	G protein
Hanks' balanced salt solution	HBSS
Inositol (1, 4, 5)-trisphosphate	$InsP_3$
Liquid junction potential	E°_L
Luteinizing hormone	LH
Membrane Voltage	V_m
Nomarski differential interference-contrast microscopy	DIC
Phorbol 12-myristate 13-acetate	TPA
Phosphate buffered saline	PBS
Phospholipase A_2	PLA_2
Phospholipase C	PLC
Prolactin	PRL
Protein kinase C	PKC
Radioimmunoassay	RIA
Salmon GnRH	sGnRH
Series resistance	R_s
Tetraethylammonia	TEA
Tetrodotoxin	TTX

Chapter 1

GENERAL INTRODUCTION

INTRODUCTION

It has become widely accepted that an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is a critical event leading to hormone secretion (Stojilkovic and Catt, 1992; Hille *et al.*, 1994, 1995). An increase in $[Ca^{2+}]_i$ may arise due to an increase in Ca^{2+} entry through plasma membrane ionic channels or the release of Ca^{2+} from intracellular stores. In pituitary cells, entry and release of Ca^{2+} is regulated by neuroendocrine stimulatory and inhibitory factors (Stojilkovic and Catt, 1992). In goldfish, gonadotropin (GTH) secretion is under stimulatory and inhibitory influence by gonadotropin releasing hormone (GnRH) and dopamine, respectively (Peter *et al.*, 1986, 1991; Chang *et al.*, 1993, 1996). Recent studies have indicated that alterations in plasma membrane ionic channel activity and release of Ca^{2+} from intracellular Ca^{2+} stores are critical to the regulation of gonadotroph cell function. However, evidence for their involvement has been based on indirect studies using pituitary fragments or mixed populations of dispersed pituitary cells (Chang *et al.*, 1993; Chang *et al.*, 1996). The primary goal of this study is to directly monitor the actions of GnRH and dopamine on plasma membrane ionic channels and mobilization of Ca^{2+} in single, identified goldfish gonadotrophs. Understanding the mechanisms of action of GnRH and dopamine at a single-cell level is critical to our understanding of how GTH-II secretion is regulated in gonadotroph cell populations.

Goldfish gonadotrophs provide an excellent model system for studying the control of GTH secretion in teleosts, as well as vertebrates for the following reasons. First, goldfish are a representative of the cyprinid family of fishes, which contains many commercially important fish species. Second, compared to other teleostean model systems, the reproductive behavior, neuroendocrine regulation and intracellular signal transduction pathways mediating GTH-II secretion from the goldfish anterior pituitary have been extensively studied (Peter *et al.*, 1991; Chang *et al.*, 1996). Third, salmon (s)-GnRH and chicken (c)-GnRH-II compete for the same class of receptors on gonadotrophs, however, sGnRH uses additional post-receptor pathways that are not involved in cGnRH-II action (Chang *et al.*, 1996). These results suggest that activation of the same receptor population by two similar peptides can lead to the differential activation of postreceptor pathways. It will be of general interest to understand how two endogenous ligands, which bind to the same population of receptors, can differentially activate separate signal transduction pathways. Fourth, dopamine activation of D_2 receptors on gonadotrophs directly inhibits basal and GnRH-stimulated GTH-II secretion, both *in vivo* and *in vitro* (Peter *et al.*, 1991; Chang *et al.*, 1993). This allows for the investigation of how inhibitory neuroendocrine factors may interact with plasma membrane ionic channels to modulate the spontaneous activity of secretory cells, as well as the activity of the stimulatory neuroendocrine regulator, GnRH. In addition, this may provide an excellent physiological model to examine the interactions of neurotransmitters and neuropeptides on exocytosis in general.

$[CA^{2+}]_i$ PATTERNS AND CONTROL OF $[CA^{2+}]i$ OSCILLATIONS

$[Ca^{2+}]_i$ patterns

Although an increase in $[Ca^{2+}]_i$ mediates agonist-induced hormone secretion in many cells, the pattern of $[Ca^{2+}]_i$ responses can be complex and vary from cell to cell. The two basic aspects of any Ca^{2+} response are how an increase in $[Ca^{2+}]_i$ occurs with respect to time and space. The temporal pattern of $[Ca^{2+}]i$ signals can be divided into oscillatory and nonoscillatory responses (Berridge, 1990). Non-oscillatory $[Ca^{2+}]_i$ responses can occur as a simple spike (Akerman *et al.*, 1991; Tomic *et al.*, 1995) or as a biphasic response, consisting of a peak and a lower amplitude plateau phase (Krsmanovic, *et al.*, 1993; Thomas *et al.*, 1996). Although there is a wide variety of oscillatory Ca^{2+} signals, they can be grouped into two general categories, sinusoidal and base-line spiking (Berridge, 1990). Sinusoidal Ca^{2+} oscillations appear as regular fluctuations in $[Ca^{2+}]_i$ about a mean value, which is often elevated above the resting values (Berridge, 1990; Bird *et al.*, 1993). Base-line spiking appears as discrete transient increases in $[Ca^{2+}]_i$ arising from a constant resting level (Berridge, 1990). In addition to the differences in the temporal patterns of Ca^{2+} responses, an increase in $[Ca^{2+}]_i$ can also display a variety of spatial patterns. In many cells there is a specific initiation point from which a Ca^{2+} wave spreads across the cell (Berridge, 1993; Rawlings, *et al.*, 1991). In others, an increase in $[Ca^{2+}]_i$ can occur uniformly throughout the cell or be restricted to a specific region, resulting in the appearance of Ca^{2+} "hot-spots" (Miller, 1988; Silver *et al.*, 1990).

Control of $[Ca^{2+}]_i$ oscillations

Oscillations in $[Ca^{2+}]_i$ can occur by the periodic opening of voltage-dependent Ca^{2+} channels or by the intermittent release of Ca^{2+} from intracellular Ca^{2+} stores (reviewed by Berridge, 1990; Berridge, 1993). In electrically excitable cells, repetitive action potential activity can lead to the periodic opening of voltage-dependent Ca^{2+} channels, resulting in the generation of $[Ca^{2+}]_i$ oscillations (Berridge, 1990). Such membrane oscillators have been observed in gonadotrophs, somatotrophs, lactotrophs, corticotrophs and GH pituitary cells.

In some pituitary cells, the membrane oscillator can be modulated by inhibitory and stimulatory neuroendocrine factors (reviewed by Stojilkovic and Catt, 1992). In electrically excitable and non-excitable cells, $[Ca^{2+}]_i$ oscillations can be generated by the periodic release of Ca^{2+} from intracellular stores. Although this response is derived by the release of Ca^{2+} from intracellular stores, Ca^{2+} entry through voltage-dependent or independent Ca^{2+} channels, is required to recharge the intracellular stores (Berridge, 1990). Initiation of $[Ca^{2+}]_i$ oscillations by neurotransmitters and hormones is due to an increase in inositol(1,4,5)-trisphosphate ($InsP_3$) levels by phospholipase C (PLC)-dependent hydrolysis of phosphatidylinositol-(4,5)bisphosphate (PIP_2). This is also associated with the generation of diacylglycerol (DAG) and the subsequent activation of protein kinase C (PKC). Two models have been proposed to describe how Ca^{2+} oscillations are generated in response to an increase in $InsP_3$ levels. In receptor-controlled models, oscillating $InsP_3$ levels are generated by various feedback effects of PKC or Ca^{2+} at the level of the plasma membrane (Berridge, 1990; Bird, *et al.*, 1993). Whereas in second messenger-controlled models, Ca^{2+} oscillations are set up in the presence of a constant level of $InsP_3$ (Berridge, 1990; Stojilkovic *et al.*, 1993). In these models, the activity of the $InsP_3$-receptor exhibits a bell-shaped response curve to an increase in $[Ca^{2+}]_i$ (Berridge, 1990).

GONADOTROPINS IN TELEOST FISH

In vertebrates, GTH secretion is regulated by multiple neuroendocrine factors, including GnRH. In tetrapods and teleost fish, GTH secreted from the pituitary is responsible for regulating the production and release of germ cells and sex steroids. The teleostean pituitary, like that of tetrapods, secretes two distinct GTHs, GTH-I and GTH-II, which are chemically and functionally similar to follicle-stimulating hormone (FSH) and luteinizing hormone (LH), of higher vertebrates, respectively (reviewed by Nagahama *et al.*, 1993). To date, two distinct GTH forms have been identified in salmonid fishes (Nagahama *et al.*, 1993), common carp (Van Der Kraak *et al.*, 1992), red seabream (Tanaka *et al.*, 1995), Mediterranean yellowtail (Hernandez *et al.*, 1995), and bonito (Koide *et al.*, 1993). Fish GTHs are members of the glycoprotein hormone family, which also includes thyroid-stimulating hormone and human chorionic GTH (Pierce and Parsons, 1981). Gonadotropin-I and GTH-II consist of two chemically distinct subunits, designated α and β, which are noncovalently bound. Both gonadotropins appear to share a similar α-subunit but have different β-subunits (Pierce and Parsons, 1981; Koide, *et al.*, 1993; Van Der Kraak *et al.*, 1992). In salmonid fishes, GTH-I stimulates ovarian growth and steroidogenesis during the early period of oocyte growth, while GTH-II stimulates final oocyte maturation (Nagahama *et al.*, 1993). These results indicate that GTH-I and GTH-II have similar biological activities as FSH and LH, respectively. In goldfish, although GTH-I and GTH-II are equally effective in stimulating the uptake of vitellogenin and inducing final oocyte maturation, GTH-II is more potent in stimulating the production of testosterone and progesterone (Van Der Kraak *et al.*, 1992). GTH-II is also the predominant form in the pituitary from prespawning female common carp (Van Der Kraak, *et al.*, 1992). In addition, the profile of GTH-II secretion throughout the seasonal reproductive cycle of goldfish is consistent with its involvement in final oocyte maturation. Taken together, these data indicate that GTH-II is more closely related to LH in tetrapods and represents the maturational GTH in goldfish. Due to the lack of a validated RIA for GTH-I in goldfish, and the use of antibodies specific for GTH-II in the verification of the cell identification technique (Chapter 2), the work in this thesis is restricted to the regulation of GTH-II.

NEUROENDOCRINE REGULATION OF GTH-II SECRETION IN GOLDFISH

Multiple stimulatory and inhibitory neuroendocrine factors act at various levels of the hypothalamic-pituitary axis to regulate GTH-II secretion from the anterior pituitary of goldfish (for reviews see, Peter *et al.*, 1991; Trudeau and Peter, 1995). The principle stimulatory and inhibitory neuroendocrine factors regulating GTH-II secretion are GnRH and dopamine, respectively. An increase in GnRH secretion and a concomitant decrease in dopaminergic inhibition are required for the induction of the preovulatory GTH-II surge and ovulation (Chang *et al.*, 1984; Peter *et al.*, 1991). Various other monoamines, amino acids, neuropeptides, steroids and growth factors are also involved in the neuroendocrine regulation of GTH-II secretion, as summarized in Fig. 1.1.

GnRH stimulation of GTH-II secretion in goldfish

In tetrapods, hypothalamic neuropeptides and neurotransmitters are released from nerve terminals in the median eminence and are transported to the anterior pituitary by a hypothalamo-hypophyseal portal vascular system (Kawano and Daikoku, 1981). A median eminence is absent in many teleosts, including goldfish (Peter *et al.*, 1990b; Kah *et al.*, 1993). Instead, hypothalamic neurosecretory fibers directly innervate the anterior pituitary (Peter *et al*. 1990; Kah *et al.*, 1993). As a result, GnRH is released from nerve terminals near to gonadotrophs to stimulate GTH-II secretion (Peter *et al.*, 1990b).

The temporal pattern of GnRH delivery to gonadotrophs in the anterior pituitary of goldfish is not yet known. This is mainly due to the technical difficulties in measuring the release of GnRH into the pituitary, as there is no hypothalamo-hypophyseal portal system. However, GnRH-induced desensitization is dependent on both the frequency and concentration of GnRH (Habibi, 1991), indicating that pulsatile GnRH release may be important in goldfish to maintain normal cell function. This has been clearly demonstrated in mammals, in which intermittent, rather than continuous, secretion of GnRH from the median eminence into the anterior pituitary is necessary to maintain normal gonadotroph cell function (Katt *et al.*, 1985; Knobil, 1992).

Since the primary structure for mammalian GnRH in porcine and ovine brains was first elucidated (Matsuo *et al.*, 1971; Burgus *et al.*, 1972), at least 9 other forms have been identified by their primary structure in vertebrates (Sherwood *et al.*, 1993; Sherwood, 1995; Powell *et al.*, 1994). Additionally, a novel GnRH form has been identified in tunicates (Sherwood, 1995) and GnRH-like immunoreactivity and bioactivity have been demonstrated in the pond snail, *Helisoma trivolvis* (Goldberg *et al.*, 1993). As in many vertebrate species, multiple forms of GnRH are found in the central nervous system of goldfish (Sherwood *et al.*, 1993). The two forms of GnRH identified in the goldfish brain and pituitary are salmon GnRH ([Trp^7, Leu^8]-GnRH; sGnRH) and chicken GnRH-II ([His^5, Trp^7, Tyr^8]-GnRH; cGnRH-II; Yu *et al.*, 1988). Salmon GnRH and cGnRH-II precursor mRNA have also been demonstrated to be expressed in the goldfish brain (Lin and Peter, 1996). Although both GnRH forms are found throughout the brain and pituitary, sGnRH is the predominant form in the forebrain and pituitary, whereas cGnRH-II predominates in the hindbrain regions (Yu *et al.*, 1988; Rosenblum *et al.*, 1994). Differential distribution of endogenous GnRH forms has also been observed in the brain of other teleosts, reptiles, amphibians and birds (Tsai and Licht, 1992; Sherwood *et al.*, 1993; Dufour *et al.*, 1993; Licht *et al.*, 1994).

The presence of multiple GnRH forms and their differential distribution in the brain and pituitary of many vertebrates suggests that different GnRHs may play different roles in the regulation of pituitary cells and other physiological functions. In goldfish, several lines of evidence implicate the involvement of both native GnRHs in the regulation of GTH-II secretion. Although the content of sGnRH is higher, both sGnRH and cGnRH-II are found in the pituitary and telencephalon-preoptic area (Yu *et al.*, 1988; Rosenblum *et al.*, 1994). The telencephalon-preoptic area is believed to be involved in the neuroendocrine regulation of pituitary cell function in goldfish (Kah *et al.*, 1993), as well as other teleosts (Amano *et al.*, 1995). In addition, these areas are believed to be targets of olfactory neurons mediating pheromonal influences on sexual behavior and GTH-II secretion (Zheng, 1996). Retrogradely-stained neurons from the goldfish pituitary were also observed in the rostral midbrain tegmentum, which has a high cGnRH-II content relative to sGnRH (Kah *et al.*, 1993; Anglade *et al.*, 1993). In addition to the presence of both native GnRHs in the telencephalon-preoptic area and pituitary, sGnRH and cGnRH-II are released from nerve terminals in the pituitary (Rosenblum *et al.*, 1994). Salmon GnRH and cGnRH-II released into the pituitary bind to the same population of receptors (Habibi and Peter, 1991) located in the plasma membrane of goldfish gonadotrophs (Cook *et al.*, 1991) to stimulate GTH-II secretion (Chang *et al.*, 1993; Chang *et al.*, 1994., Chang *et al.*, 1996). Although these results implicate the involvement of both native GnRHs in the neuroendocrine regulation of pituitary cell function, they do not discount other possible functions of cGnRH-II. For example, cGnRH-II has been demonstrated to play a neuromodulatory role in some vertebrates (Elmslie *et al.*, 1990; Kah *et al.*, 1993; Sherwood *et al.*, 1993). The participation of more than one GnRH form in the regulation of GTH-II-secretion is not a universal phenomenon among teleosts or vertebrates in general (Kah *et al.*, 1993; Sherwood *et al.*, 1993). However, in at least one other vertebrate group, *Rana* frogs, the two endogenous

GnRHs, mGnRH and cGnRH-II, have been implicated in the physiological regulation of GTH secretion (Licht *et al.*, 1994).

Dopamine inhibition of GTH-II secretion in goldfish

The presence of a gonadotropin-release inhibitory factor (GRIF) in goldfish was first suggested by Peter *et al.* (1978) on the basis of brain lesioning experiments (reviewed in Peter *et al.*, 1986). Dopamine was later identified to be the GRIF in goldfish (Chang and Peter, 1983; Chang *et al.*, 1983). Although dopamine exerts tonic inhibitory control over GTH-II secretion throughout the seasonal reproductive cycle, dopaminergic inhibition of GTH-II secretion is greatest in prespawning fish (Sokolowska *et al.*, 1985b). This allows for the maintenance of relatively low circulating GTH-II levels in response to increasing pituitary responsiveness to GnRH (Peter *et al.*, 1986; Habibi *et al.*, 1989; Omeljaniuk, *et al.*, 1989; Trudeau *et al.*, 1993a). In addition, the removal of inhibitory dopamine tone prior to, or in conjunction with, an increase in GnRH stimulation is required to induce the rapid increase in circulating GTH-II levels necessary to trigger ovulation (Chang and Peter, 1983).

In goldfish, dopamine inhibits GTH-II secretion at various levels of the hypothalamic-pituitary axis. Dopamine inhibits spontaneous GnRH secretion from the preoptic-anterior hypothalamus by the activation of dopamine D_1-like receptors (Yu *et al.*, 1991; Yu and Peter, 1992). Dopamine also inhibits GTH-II secretion from nerve terminals in the pituitary (Yu and Peter, 1992), presumably by axo-axonal interactions of dopamine and GnRH nerve terminals. In contrast to the situation in the preoptic-anterior hypothalamus, dopamine D_2-like receptors mediate the actions of dopamine on GnRH nerve terminals in the pituitary (Yu and Peter, 1992). Finally, dopamine directly inhibits spontaneous and GnRH-stimulated GTH-II secretion from gonadotrophs via dopamine-D_2 like receptors (Chang *et al.*, 1984; Peter, *et al.*, 1991; Chang *et al.*, 1993).

In goldfish, dopaminergic neurons innervate the anterior pituitary (Kah *et al.*, 1993). Brain lesioning and immunocytochemical studies indicated that dopaminergic fibers originate in the anteroventral preoptic region and pass through the ventral anterior hypothalamic area and pituitary stalk to reach the anterior pituitary, where they terminate near to gonadotrophs (Peter *et al.*, 1978; Peter and Paulencu, 1980; Peter *et al.*, 1986; Kah *et al.*, 1993). The close association of dopaminergic and GnRH pathways in the goldfish forebrain and pituitary (Kah *et al.*, 1993) is consistent with the proposed inhibitory actions of dopamine on GnRH secretion in these areas (Yu *et al.*, 1991; Yu and Peter, 1992; Peter *et al.*, 1991; Chang *et al.*, 1993). Moreover, dopaminergic neurons receive GnRH innervation, suggesting that GnRH may also act on the dopaminergic pathway to further regulate GnRH secretion (Kah *et al.*, 1993). This suggests that GnRH and dopaminergic neuronal networks act as an integrated unit to control GTH-II secretion.

Other neuroendocrine regulators of GTH-II secretion in goldfish

In addition to dopamine, other monoamines regulate GnRH and GTH-II secretion. In goldfish, norepinephrine and α-adrenergic agonists increase serum GTH-II levels (Chang *et al.*, 1983; Chang and Peter, 1984), indicating a stimulatory α-adrenergic influence on GTH-II secretion *in vivo. In vitro,* norepinephrine acts through α_1-adrenergic receptors to stimulate GnRH secretion from preoptic-anterior hypothalamic slices (Yu and Peter, 1992) as well as GTH-II secretion from dispersed goldfish pituitary cells (Chang *et al.*, 1991b). Serotonin also stimulates GnRH secretion from preoptic-hypothalamic and pituitary fragments (Yu *et al.*, 1991), as well as GTH-II secretion from dispersed goldfish pituitary cells (Wong, 1995). The stimulatory actions of serotonin are mediated by 5-HT_2-like receptors (Somoza *et al.*, 1988; Wong, 1995). These results indicate that norepinephrine and serotonin act at various levels of the hypothalamic-pituitary axis of goldfish to exert both direct and indirect stimulatory influences over GTH-II secretion.

Amino acids, neuropeptides and growth factors also stimulate GTH-II secretion in goldfish. The amino acid neurotransmitters, L-glutamine, taurine and γ-aminobutryic acid (GABA) stimulate GTH-II secretion (Trudeau and Peter, 1995). These stimulatory actions are mediated by indirect actions through GnRH and dopaminergic neuronal pathways. For example, GABA increases GnRH secretion and decreases dopaminergic activity, but does not stimulate GTH-II secretion from dispersed goldfish pituitary cells, arguing against a direct action of GABA on gonadotroph cell function (Trudeau and Peter, 1995). In contrast, neuropeptide Y (NPY), inhibin and activin stimulate GTH-II secretion at the level of the gonadotroph (Peter, *et al.*, 1991; Ge, *et al.*, 1992; Trudeau and Peter, 1995). NPY also stimulates the release of GnRH from nerve terminals in the brain and pituitary, which in turn stimulates GTH-II secretion (Peng *et al.*, 1993). Other peptidergic stimulatory factors include cholecystokinin and endogenous opioid peptides (Peter, *et al.*, 1991; Trudeau and Peter, 1995).

Gonadal steroids exert negative and positive feedback effects on GTH-II secretion at various levels of the brain-pituitary axis (Peter *et al.*, 1991; Trudeau and Peter, 1995). In sexually regressed goldfish, testosterone elicits a decrease in dopamine turnover in the telencephalon-preoptic area, which may represent a decrease in inhibitory dopamine tone. In contrast, in sexually recrudescent goldfish, gonadal steroids increase dopamine turnover in the telencephalon-preoptic area to augment inhibitory dopamine tone (Trudeau *et al.*, 1993a). Additionally, in sexually regressed goldfish, testosterone enhances the stimulatory action of GABA on GTH-II secretion, while in sexually recrudescent goldfish, estradiol decreases the action of GABA (Trudeau *et al.*, 1993b). Thus, gonadal steroids exert both positive and negative feedback effects on dopaminergic and GABAnergic pathways in the brain, depending on the reproductive status of the fish. In addition to the actions of sex steroids at the level of the brain, they also exert negative feedback effects at the level of the pituitary by increasing dopamine inhibitory tone (Trudeau, *et al.*, 1993a). In contrast, gonadal steroids can also exert positive feedback effects by acting directly on gonadotrophs to increase their responsiveness to GnRH. This is in part due to an increase in GnRH receptor capacity (Habibi and Peter, 1991). Recent studies also indicate that sex steroids act directly on second messenger systems to augment GnRH-stimulated GTH-II secretion (Lo *et al.*, 1995; Chang *et al.*, 1996).

GNRH-RECEPTORS AND INTRACELLULAR SIGNALING PATHWAYS IN GOLDFISH GONADOTROPHS

Although several neuroendocrine factors act directly on gonadotrophs to regulate GTH-II secretion, the receptors and associated second messenger pathways are perhaps best characterized for GnRH-stimulated GTH-II secretion. A summary of the signal transduction pathways mediating GnRH-stimulated GTH-II secretion in the goldfish pituitary are outlined below and are summarized in Fig. 1.2.

GnRH receptors in goldfish

The actions of peptides, neuropeptides and neurotransmitters on cellular activity of their target cells are mediated by specific receptors located in the plasma membrane. In vertebrates, including goldfish, the binding of GnRH to GnRH receptors in the plasma membrane of gonadotrophs initiates a series of biochemical events resulting in the synthesis and secretion of GTH-II (Chang *et al.*, 1994; Stojilkovic *et al.*, 1994; Chang *et al.*, 1996). Based on radio-receptor assays, it has been shown that the goldfish pituitary contains two classes of GnRH binding sites: a high-affinity/low-capacity binding site and a low-affinity/high-capacity binding site (Habibi *et al.*, 1987). The GTH-II-releasing actions of sGnRH and cGnRH-II are mediated by the high-affinity/low-capacity GnRH binding sites (Habibi and Peter, 1991). Several lines of evidence indicate that sGnRH and cGnRH-II act on the same population of receptors in gonadotrophs to stimulate GTH-II secretion. Both sGnRH and cGnRH-II displace bound ^{125}I-[D-Arg^6, Pro^9-N-ethylamide]-sGnRH from crude pituitary membrane preparations (Habibi and Peter, 1991), as well as avidin-gold labeled [D-Arg^6, Pro^9-N-ethylamide]-sGnRH from immunohistochemically identified goldfish gonadotrophs (Cook *et al.*, 1991). The GTH-II release response to maximally effective doses of sGnRH and cGnRH-II are not additive (Chang *et al.*, 1993). sGnRH- and cGnRH-II-stimulated GTH-II secretion were equally inhibited by competitive antagonists (Murthy and Peter, 1994). In the continued presence of sGnRH or cGnRH-II, further stimulation by either GnRH is inhibited, suggesting that there is cross desensitization of the GTH-II release response by either of the GnRHs (Murthy and Peter, 1994). Prolonged treatment with either GnRH results in a similar reduction in the GTH-II release response to subsequent applications of sGnRH or cGnRH-II (Murthy and Peter, 1994). Taken together, these studies strongly indicate the involvement of a single population of GnRH receptors in the mediation of sGnRH and cGnRH-II action. Definitive proof of a single GnRH receptor type mediating the actions of both GnRHs awaits the cloning of GnRH receptors from the goldfish pituitary.

GnRH binding sites and GnRH receptors have also been identified in other pituitary cell types and extrapituitary tissues. Most notably, GnRH receptors have been identified in immunohistochemically-identified goldfish somatotrophs (Cook *et al.*, 1991), which is consistent with the involvement of sGnRH and cGnRH-II in regulating GH secretion from the pituitary of goldfish and other related species (Chang *et al.*, 1994; Peter and Marchant, 1995). Salmon-GnRH and cGnRH-II bind to receptors in the plasma membrane of somatotrophs to stimulate GH secretion. These receptors may be distinct from those found in gonadotrophs. Photoaffinity labelling studies revealed a 51 000-M_r protein which appeared as two closely associated bands (Habibi *et al.*, 1990) and may represent distinct populations of GnRH receptors in gonadotrophs and somatotrophs. In support of this view, certain GnRH analogs function as antagonists for GTH-II secretion but are effective agonists for GH secretion (Murthy and Peter, 1994). In addition to the pituitary, GnRH binding sites have also been identified in the liver, mature ovary, testis and kidney of goldfish (Habibi and Pati, 1993).

G-protein and phospholipase C involvement in GnRH action

Heterotrimeric guanine nucleotide binding proteins (G-proteins) link receptors to effectors in a wide variety of cell types, including pituitary cells (Hille *et al.*, 1994). G-proteins are composed of three subunits including α, β and γ, of which the β and γ subunits are tightly but non-covalently associated to form a βγ-complex (Spiegel *et al.*, 1992). Receptor activation by ligand binding, promotes the release of GDP from the α-subunit, allowing for the binding of GTP. Binding of GTP causes a conformational change in the α-subunit, which in turn leads to the dissociation of the G-protein from its receptor and separation of the α-subunit from the βγ-complex. The GTP-bound α-subunit and the βγ-complex are then free to activate various effectors, such as phospholipase C (PLC) isozymes (Berridge, 1993) or ionic channels (Hille, 1992; Boland and Bean, 1993). The intrinsic GTPase activity of the α-subunit causes the hydrolysis of bound GTP to GDP with the liberation of inorganic phosphate, resulting in the dissociation of the α-subunit from the effector and its reassociation with a $\beta\gamma$-complex (reviewed in Spiegel *et al.*, 1992). In this way, G-proteins allow receptors to turn "on" and "off" various effectors to mediate a wide range of cellular responses, including mobilization of intracellular Ca^{2+} (Berridge, 1993) and regulation of ionic channel activity (Hille, 1992; Clapham, 1994). In goldfish, preliminary studies have indicated the presence of cholera toxin- and pertussis toxin-sensitive G proteins (Chang *et al.*, 1993). However, whether other G-proteins, which are not sensitive to these toxins, are present remains to be determined.

In some cells, G-protein activation stimulates the hydrolysis of PIP_2 by PLC, which in turn leads to the generation of $InsP_3$ and DAG (Berridge, 1993). In goldfish, addition of PLC stimulates GTH-II secretion (Chang *et al.*, 1991c), suggesting that PLC-dependent pathways are present in goldfish gonadotrophs. Moreover, sGnRH stimulates PLC-mediated hydrolysis of phosphoinositides, resulting in the production of major inositol phosphate groups, including $InsP_1$, $InsP_2$, $InsP_3$ and other higher InsPs (Chang *et al.*, 1995), as well as the formation of DAG (Chang *et al.*, 1993; Chang *et al.*, 1996). Taken together, these results suggest the involvement of PLC in the mediation of GnRH-stimulated GTH-II secretion in goldfish. It is important to note that unlike sGnRH, cGnRH-II increases $InsP_2$ production but does not stimulate the accumulation of $InsP_3$ isoforms, suggesting that sGnRH and cGnRH-II may interact with different PLC isozymes or InsPs metabolizing enzymes (Chang *et al.*, 1995). Further studies are required to demonstrate the direct involvement of G-proteins, and the G-protein and PLC subtypes involved in the mediation of GnRH-stimulated GTH-II secretion.

Role of protein kinase C and Ca^{2+} in mediating GnRH action

In neuronal and endocrine cells, an increase in $[Ca^{2+}]_i$ is widely regarded to be the final common pathway mediating secretion. Accordingly, a rise in $[Ca^{2+}]_i$ has been demonstrated to be central to the mediation of pituitary hormone secretion in general (Stojilkovic and Catt, 1992), and LH secretion in particular (Stojilkovic *et al.*, 1994). A rise in $[Ca^{2+}]_i$ can originate from two general Ca^{2+} sources; extracellular Ca^{2+} entry and intracellular Ca^{2+} release. Mobilization of extracellular Ca^{2+} is mediated by a variety of pathways, including voltage-dependent Ca^{2+} channels and receptor-operated Ca^{2+} channels. On the other hand, mobilization of intracellular Ca^{2+} is mediated by $InsP_3$ and ryanodine receptors (Berridge, 1993). It has been proposed that in goldfish gonadotrophs, GnRH-stimulates a rise in $[Ca^{2+}]_i$ by mobilizing extracellular entry and intracellular Ca^{2+} release (Jobin and Chang, 1992a; Jobin and Chang, 1992b; Chang *et al.*, 1996).

Extracellular Ca^{2+} availability is critical to the normal functioning of goldfish gonadotrophs. During 2-hr static incubation experiments (prolonged hormone release response), removal of extracellular Ca^{2+} attenuated the GTH-II response to sGnRH and abolished cGnRH-II-stimulated GTH-II secretion (Jobin and Chang, 1992a). Similarly, in perifusion experiments (short-term hormone release response), removal of extracellular Ca^{2+} reduced both sGnRH- and cGnRH-II stimulated GTH-II secretion (Jobin *et al.*, 1996). Taken together, these results demonstrate that both short-term and prolonged GnRH action is dependent on the availability of extracellular Ca^{2+}; further implicating extracellular Ca^{2+} in the mediation of GTH-II secretion, sGnRH and cGnRH-II increased $[Ca^{2+}]_i$ in mixed populations of dispersed goldfish pituitary (Jobin and Chang, 1992b). Consistent with hormone release studies, sGnRH-stimulated increases in $[Ca^{2+}]_i$ were reduced by removal of extracellular Ca^{2+}, while the responses to cGnRH-II- were totally abolished. It should, however, be mentioned that the intracellular Ca^{2+} response to sGnRH and cGnRH-II cannot be attributed entirely to gonadotrophs, as mixed populations of dispersed goldfish pituitary cells were used. Nevertheless, results from both hormone-release and $[Ca^{2+}]_i$ measurement studies indicate that extracellular Ca^{2+} is involved in the actions of sGnRH and cGnRH-II. Moreover, compared to sGnRH, cGnRH-II action is more sensitive to removal of extracellular Ca^{2+}, suggesting that there are possible differences in the signal transduction pathways mediating the two native GnRHs. In particular, sGnRH action appears to involve the mobilization of Ca^{2+} from intracellular stores. These results are consistent with the ability of sGnRH, but not cGnRH-II, to increase $InsP_3$ levels (Chang *et al.*, 1995).

Hormone release and $[Ca^{2+}]_i$ measurement studies on dispersed goldfish pituitary cells have suggested that the extracellular Ca^{2+}-dependence of GnRH action is in part due to the involvement of voltage-dependent Ca^{2+} channels. Application of Co^{2+}, an inorganic voltage-dependent Ca^{2+} channel blocker, as well as dihydropyridine and phenylalkylamine inhibitors of voltage-dependent Ca^{2+} channels, attenuate sGnRH- and cGnRH-II-stimulated GTH-II secretion in static incubation and perifusion experiments (Jobin and Chang, 1992a; Jobin and Chang, 1992b; Jobin *et al.*, 1996). Similarly, cGnRH-II-stimulated increases in $[Ca^{2+}]_i$ is reduced by nicardipine, a dihydropyridine voltage-dependent Ca^{2+} channel antagonist (Chang *et al.*, 1996). Conversely, Bay K 8644, a dihydropyridine voltage-dependent Ca^{2+} channel agonist, stimulates basal GTH-II secretion, and augments sGnRH- and cGnRH-II-induced GTH-II secretion (Chang *et al.*, 1996; Jobin *et al.*, 1996). These results strongly support the participation of voltage-dependent Ca^{2+} channels in GnRH-stimulated GTH-II secretion from the goldfish pituitary. However, the voltage-dependent characteristics of the Ca^{2+} channels present in goldfish gonadotrophs, as well as the direct demonstration of their involvement in mediating the actions of GnRH awaits patch-clamp studies on single identified goldfish gonadotrophs.

The involvement of PKC in mediating GnRH action in mammalian gonadotrophs has been a subject of much debate. Some have suggested that PKC is obligatory for secretion while others have suggested that it plays a modulatory role (Davidson *et al.*, 1990; Stojilkovic *et al.*, 1991; Stojilkovic and Catt, 1992; Jobin *et al.*, 1995; Tse *et al.*, 1995). It has also been proposed that PKC is not involved in the secretory response to GnRH (McArdle *et al.*, 1987). In goldfish, several lines of evidence strongly support the involvement of PKC in mediating both short-term and prolonged stimulation of GTH-II secretion by sGnRH and cGnRH-II (Chang *et al.*, 1993; Chang *et al.*, 1996). Application of synthetic diacylglycerol (DAG) or activation of PKC by tumor-promoting phorbol esters stimulates GTH-II secretion (Chang *et al.*, 1991a). Specific inhibitors of PKC reduce the GTH-II-release response to synthetic DAG, phorbol esters, sGnRH and cGnRH-II (Chang *et al.*, 1991a; Jobin *et al.*, 1996). The maximal GTH-II response to GnRH and that of phorbol esters or synthetic DAG are not additive in both static incubation and perifusion experiments (Jobin *et al.*, 1996). In cells depleted of PKC, both the short-term and prolonged hormone release response to sGnRH and cGnRH-II, as well as the super-active GnRH analog, [D-Ala^6, Pro^9,-Net]-sGnRH, are markedly attenuated (Jobin and Chang, 1993; Jobin *et al.*, 1993). The reduction in GnRH-stimulated GTH-II secretion in PKC depleted cells is not due to a reduction in the GTH-II releasable pool or an impairment of the exocytotic machinery, as elevations in cyclic adenosine-3′,5′-monophosphate (cAMP) were still able to stimulate GTH-II secretion (Jobin *et al.*, 1993).

In goldfish, hormone release and [Ca^{2+}] measurement studies from mixed populations of dispersed goldfish pituitary cells suggest that PKC activates voltage-dependent Ca^{2+} channels to induce extracellular Ca^{2+} influx and the subsequent secretion of GTH-II (Chang *et al.*, 1993; Chang and Jobin, 1994; Chang *et al.*, 1996). Protein kinase C-stimulated GTH-II secretion is reduced by removal of extracellular Ca^{2+} or by addition of voltage-dependent Ca^{2+} channel blockers (Chang *et al.*, 1991a; Jobin and Chang, 1992b). Conversely, Bay K 8644 and membrane depolarizations by high concentrations of extracellular K^+ potentiate PKC-stimulated GTH-II secretion (Jobin and Chang, 1992b; Jobin *et al.*, 1996). Activators of PKC stimulate an increase in $[Ca^{2+}]_i$ in mixed populations of goldfish pituitary cells that is reduced or abolished by the removal of extracellular Ca^{2+} or by the application of dihydropyridine voltage-dependent Ca^{2+} channel antagonists (Jobin and Chang, 1992b; Chang *et al.*, 1996). Finally, staurosporine, a PKC inhibitor, reduces PKC-, and GnRH-stimulated increases in $[Ca^{2+}]_i$ in mixed populations of dispersed goldfish pituitary cells (Jobin and Chang, 1992b; Chang *et al.*, 1996). Whether PKC directly activates voltage-dependent Ca^{2+} channels in goldfish gonadotrophs remains to be investigated.

Role of calmodulin in goldfish gonadotrophs

Calmodulin is an ubiquitous Ca^{2+}-binding protein which is activated by an increase in $[Ca^{2+}]_i$. Calmodulin affects a wide variety of enzymes, including Ca^{2+}-calmodulin-dependent protein kinase, adenylate cyclase, cyclic nucleotide phosphodiesterase, and synaptic vesicle-associated phosphoproteins (Hanson and Schulman, 1992; Benfenati *et al.*, 1992). In goldfish gonadotrophs, calmodulin and calmodulin kinase II do not mediate the short-term actions of sGnRH or cGnRH-II; however, they participate in prolonged GnRH-stimulated GTH-II secretion (Jobin *et al.*, 1996). Whether calmodulin and calmodulin-dependent protein kinase are involved in stimulus-transcription coupling or other signaling pathways in goldfish gonadotrophs remains to be elucidated.

Role of phospholipase A_2 and arachidonic acid in goldfish gonadotrophs

Arachidonic acid and several of its lipoxygenase or epoxygenase products stimulate LH secretion in rats (Naor *et al.*, 1985). The participation of arachidonic acid metabolism during GnRH-stimulated GTH-II secretion has also been demonstrated in goldfish (Chang *et al.*, 1989; Chang *et al.*, 1993; Chang *et al.*, 1996). In goldfish, inhibitors of phospholipase A_2 (PLA_2) and lipoxygenase reduce sGnRH-stimulated GTH-II secretion (Chang *et al.*, 1994). In contrast, inhibitors of DAG lipase or cyclooxygenase do not affect sGnRH-stimulated GTH-II secretion (Chang *et al.*, 1994; Chang *et al.*, 1996). These results indicate that sGnRH mobilizes arachidonic acid via activation of PLA_2 and stimulates its subsequent metabolism by the lipoxygenase enzyme (Chang *et al.*, 1991c). Additionally, arachidonic acid-stimulated GTH-II secretion is independent of extracellular Ca^{2+} availability (Chang *et al.*, 1991c), which is consistent with the incomplete inhibition of sGnRH-stimulated GTH-II secretion by the removal of extracellular Ca^{2+} (Jobin and Chang, 1992a). In contrast, cGnRH-II stimulated GTH-II secretion was not affected by inhibitors of PLA_2, DAG lipase, lipoxygenase or cyclooxygenase (Chang *et al.*, 1994; Chang *et al.*, 1996), indicating that these pathways are not involved in mediating the action of cGnRH-II. The participation of PLA_2 and arachidonic acid in sGnRH-, but not cGnRH-II-stimulated GTH-II secretion, provides further evidence that the mechanisms of action between the two native GnRHs are not the same.

Possible role of Cyclic AMP in goldfish gonadotrophs

In goldfish, addition of the adenylate cyclase activator, forskolin, or the phosphodiesterase inhibitor, IBMX, elevate cAMP production as well as GTH-II secretion (Chang *et al.*, 1992). However, cAMP does not appear to be directly involved in the GTH-II release response to sGnRH or cGnRH-II, as neither stimulates an increase in cAMP production (Chang *et al.*, 1992). Additionally, sGnRH- and cGnRH-II-stimulated GTH-II secretion is not affected by inhibitors of cAMP-dependent protein kinase (Jobin *et al.*, 1996). Moreover, the GTH-II release response to treatments which elevate cAMP are additive or synergistic to sGnRH- and cGnRH-II-stimulated GTH-II secretion (Chang *et al.*, 1992), as well as arachidonic acid- and PKC-induced GTH-II responses (Jobin *et al.*, 1996). Taken together, these results indicate that cAMP and cAMP-dependent pathways are not activated during GnRH action in goldfish gonadotrophs.

Although cAMP may not be directly involved in the mediation of GnRH action in goldfish, it may act to positively modulate GnRH-stimulated GTH-II secretion. In some normal and clonal pituitary cell types, cAMP interacts with Ca^{2+}- and PKC-dependent signal transduction pathways to mediate hormone secretion (Mollard *et al.*, 1992; Waring and Turgeon, 1992; Chuang *et al.*, 1993; Rawlings *et al.*, 1993; Gross *et al.*, 1994). It has also been suggested that cAMP mobilizes LH from a non-releasable to a releasable pool to maintain GnRH-stimulated LH secretion (Janovick and Conn, 1993). In goldfish, stimulation of cAMP-dependent pathways sensitized the hormone response to PKC (Chang *et al.*, 1996). Moreover, elevations in cytosolic cAMP levels enhanced sGnRH- and cGnRH-II-stimulated GTH-II secretion (Chang *et al.*, 1992). As Ca^{2+} influx through voltage-dependent Ca^{2+} channels is likely involved in the actions of PKC and GnRH, it is possible that the sensitizing action of cAMP on GTH-II secretion is due to positive modulation of voltage-dependent Ca^{2+} channels.

Voltage-dependent Ca^{2+} channels contain cAMP-dependent phosphorylation sites and are modulated by cAMP in different cell types (Hurwitz 1986; Tsien *et al.*, 1988; Mollard, *et al.*, 1992) including pituitary cells lines (Gross *et al.*, 1994). Whether cAMP alters voltage-dependent Ca^{2+} channel activity awaits studies on single identified goldfish gonadotrophs using patch-clamp recording techniques.

MECHANISMS OF DOPAMINE ACTION IN GOLDFISH GONADOTROPHS

Compared to GnRH actions, the intracellular signal transduction pathways mediating the direct inhibitory actions of dopamine on goldfish gonadotrophs are not as well characterized (Fig 1.2). However, one way that dopamine inhibits GTH-II secretion is by acting on GnRH receptors. In *in vivo* studies, injection of dopamine D_2 antagonists increased the capacity, but did not change the affinity, of both the high and low affinity GnRH binding sites (Habibi and Peter, 1991). Similar results were observed in *in vitro* studies on goldfish pituitary fragments using the general dopamine agonist, apomorphine (Habibi and Peter, 1991). These studies suggest that dopamine causes a down-regulation of the high and low affinity GnRH binding sites in goldfish gonadotrophs. However, the cellular mechanisms underlying this response remain to be elucidated. Since GnRH receptors cloned from mice confirm potential PKC- and cAMP-dependent phosphorylation sites (Stojilkovic *et al.*, 1994), similar sites on goldfish GnRH receptors may be involved. This possibility can be tested once these receptors have been cloned.

Given the importance of voltage-dependent Ca^{2+} channels in the regulation of GnRH-stimulated GTH-II secretion in goldfish, they are an obvious target for the inhibitory actions of dopamine. However, there is little known about the interactions between dopamine and voltage-dependent Ca^{2+} channels in teleostean gonadotrophs. Preliminary studies suggest that dopamine D_2 receptor agonists reduce cGnRH-II-induced increases in $[Ca^{2+}]_i$ from mixed populations of dispersed goldfish pituitary cells (Chang *et al.*, 1993). Also, dopamine D_2 receptor agonists reduce PKC-stimulated GTH-II secretion (Jobin and Chang, 1993). These results suggest that dopamine may inhibit GTH-II secretion by altering the activity of PKC or voltage-dependent Ca^{2+} channels in goldfish gonadotrophs. In rat lactotrophs (Lledo *et al.*, 1990) and melanotrophs (Keja *et al.*, 1992), as well as frog melanotrophs (Valentijn *et al.*, 1993), dopamine reduces Ca^{2+} influx through voltage-dependent Ca^{2+} channels to inhibit hormone secretion. Whether a similar pathway contributes to the inhibition of GTH-II secretion by dopamine in goldfish can best be determined in electrophysiological studies of identified goldfish gonadotrophs.

GNRH AND DOPAMINE MECHANISMS OF ACTION IN GONADOTROPHS FROM OTHER FISH.

As in goldfish, hypothalamic neurosecretory fibers deliver GnRH directly to the anterior pituitary of the African catfish (Peute *et al.*, 1987), seabass (Moons *et al.*, 1989), moly (Batten *et al.*, 1990), dwarf gouramie (Oka and Ichikawa, 1990), steelhead trout (Parhar *et al.*, 1994) and Atlantic salmon (Holmquist and Ekstrom, 1995). Similar to the situation in goldfish, entry of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels plays an important role in mediating the actions of GnRH in tilapia (Levavi-Sivan and Yaron, 1989), African catfish (van Asselt *et al.*, 1989; Rebers *et al.*, 1995), trout (Flores, 1995), and common carp (Mikolajczyk *et al.*, 1990). Moreover, in African catfish and trout gonadotrophs, GnRH stimulates an increase in $[Ca^{2+}]_i$ (Flores, 1995; Rebers, *et al.*, 1995).

However, unlike the situation in goldfish, cAMP is involved in mediating GnRH-stimulated GTH-II secretion in tilapia (Levavi-Sivane and Yaron, 1992; Levavi-Sivan *et al.*, 1995), African catfish (van Asselt 1989) and Indian murrel (Mukhopadhyay *et al.*, 1995). Nevertheless, a rise in $[Ca^{2+}]_i$ due to Ca^{2+} entry through voltage-dependent Ca^{2+} channels appears to be a common pathway in mediating GnRH-stimulated GTH-II secretion in teleosts.

Dopamine also acts as a GRIF in African catfish (de Leeuw *et al.*, 1985; de Leeuw *et al.*, 1986), tilapia (Gissis *et al.*, 1988), common carp (Lin *et al.*, 1988), Chinese loach (Lin *et al.*, 1988), European eel (Dufour *et al.*, 1988), rainbow trout and brown trout (Billard *et al.*, 1984). However, dopamine-induced inhibition of GTH-II secretion is less prevalent in coho salmon (Van Der Kraak *et al.*, 1986), and is absent in the Atlantic croaker (Copeland and Thomas, 1989). In recent studies from tilapia, it was suggested that the inhibitory actions of dopamine are in part due to a reduction in PKC-stimulated GTH-II secretion (Levavi-Sivan *et al.*, 1995). It was also suggested that the inhibitory actions of dopamine occur distal to Ca^{2+} influx and may occur at the level of the intracellular Ca^{2+} stores (Levavi-Sivan *et al.*, 1995). However, the authors used Ca^{2+} ionophores to investigate the possible actions of dopamine on Ca^{2+} influx and possible influences of dopamine on Ca^{2+} entry through voltage-dependent Ca^{2+} channels might have been missed. In support of this view, both voltage-dependent Ca^{2+} channel antagonists and dopamine reduced basal GTH-II secretion (Levavi-Sivan *et al.*, 1995; Levavi-Sivan and Yaron, 1993), suggesting that voltage-dependent Ca^{2+} channels may be a target for dopamine inhibition in tilapia gonadotrophs.

PLASMA MEMBRANE EVENTS AND [CA^{2+}]$_i$ RESPONSES IN UNSTIMULATED AND GNRH-STIMULATED RAT GONADOTROPHS.

Alterations in electrical membrane activity and [Ca^{2+}]$_i$ are important events in the regulation of secretory responses in endocrine cells, including goldfish gonadotrophs. To understand the participation of the plasma membrane and intracellular Ca^{2+} stores during basal and GnRH-stimulated GTH-II secretion in teleosts, it is helpful to first understand their activity in the well-characterized rat gonadotroph cell model. Therefore, the involvement of the plasma membrane and intracellular Ca^{2+} stores in both unstimulated and GnRH-stimulated rat gonadotrophs will be briefly reviewed (see also Stojilkovic and Catt, 1992, 1995; Hille *et al.*, 1994, 1995; Stojilkovic *et al.*, 1994).

Unstimulated rat gonadotrophs.

Rat gonadotrophs, like all pituitary cells, are electrically excitable as they exhibit voltage-dependent action potential activity (Stojilkovic and Catt, 1992). Multiple classes of voltage-dependent Ca^{2+} channels, tetrodotoxin-sensitive Na$^+$ channels, multiple voltage-dependent K$^+$ channels subtypes, apamin-sensitive Ca^{2+}-activated K$^+$ channels and Cl$^-$ channels have been identified in rat gonadotrophs (Stojilkovic and Catt, 1992; Tse and Hille, 1993). The two classes of voltage-dependent Ca^{2+} channels present in female rat gonadotrophs are similar to the L (long-lasting)- and T (transient)-type Ca^{2+} channels characterized in other cells (Tsien *et al.*, 1988). In gonadotrophs from male rats, L-type Ca^{2+} channels and a type which is not N- or T-like are present (Tse and Hille, 1993).

Spontaneous action potential activity is observed in unstimulated female rat gonadotrophs (Stojilkovic and Catt, 1992). In these cells, action potential spikes are fired when a slow spontaneous depolarization reaches a threshold; this is followed by a sharp hyperpolarization. These action potentials are not sensitive to the voltage-dependent Na^+ channel blocker, tetrodotoxin, but are reduced by the addition of dihydropyridine voltage-dependent Ca^{2+} channel antagonists (Stojilkovic *et al.*, 1992). This indicates that spontaneous action potential activity in these cells is due to the intermittent opening of dihydropyridine-sensitive Ca^{2+} channels. The spike phase is initiated by the activation of T-type voltage-dependent Ca^{2+} channels but is predominantly due to Ca^{2+} influx through L-type Ca^{2+} channels. Accordingly, spontaneous action potential activity in these cells is accompanied by localized increases in $[Ca^{2+}]_i$ at the sites of Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Iida *et al.*, 1991, Li *et al.*, 1995). This increase in $[Ca^{2+}]_i$ activates Ca^{2+}-activated K^+-channels, which together with voltage-dependent K^+ channels, are responsible for the termination of the action potential spike and for the after-hyperpolarization between two spikes. The slow decrease in $[Ca^{2+}]_i$ following the termination of the spike leads to the closing of Ca^{2+}-activated K^+-channels and the slow depolarization of the membrane. Thus, the coordinated actions of voltage-dependent Ca^{2+} and K^+ channels, as well as Ca^{2+}-activated K^+-channels, provide the basis for spontaneous action potential activity (Li *et al.*, 1995). The frequency of spontaneous action potential firing is determined by the magnitude and duration of the Ca^{2+}-activated K^+-channel-dependent after-hyperpolarizations (Li *et al.*, 1995). Unlike the spontaneous action potential activity observed in female rat gonadotrophs, none has been observed in male rat gonadotrophs (Tse and Hille, 1992, 1993). It has been suggested that this may be due to differences in the magnitude of voltage-dependent K^+ currents or voltage-dependent Ca^{2+} currents between male and female rat gonadotrophs (Li *et al.*, 1995). In addition, prior identification of ovine gonadotrophs by plaque assay results in electrically silent cells compared to those from enriched cell populations (Heyward *et al.*, 1993). This suggests that extensive pretreatment can also alter electrical membrane activity in some gonadotroph cell preparations.

Unlike rat somatotrophs and lactotrophs, the action-potential-driven increase in $[Ca^{2+}]_i$ in rat gonadotrophs appears to be insufficient to stimulate LH secretion (Stojilkovic and Catt, 1992). Basal hormone secretion in rat gonadotrophs is relatively low and insensitive to manipulations of extracellular Ca^{2+} entry compared to lactotrophs and somatotrophs (Stojilkovic *et al.*, 1988). This distinction between somatotrophs/lactotrophs and gonadotrophs in rats may reflect the differences in their neuroendocrine regulation. For example, like gonadotrophs in goldfish and other teleosts (Peter *et al.*, 1986; Peter *et al.*, 1991), somatotrophs and lactotrophs in rats are under the influence of both stimulatory and inhibitory hypothalamic neuroendocrine factors (de la Escalera and Weiner, 1992; Stojilkovic and Catt, 1992; Chen *et al.*, 1994b). In contrast, rat gonadotrophs are predominately regulated by the stimulatory hypothalamic neuroendocrine factor, GnRH (Stojilkovic and Catt, 1992, Stojilkovic *et al.*, 1994).

GnRH-stimulated rat gonadotrophs.

In rat gonadotrophs, GnRH binding to its receptor activates pertussis toxin-insensitive G-proteins (Tse and Hille, 1992), specifically G_q and/or G_{11} (Hsieh and Martin, 1992). This leads to the activation of PLC (Zheng *et al.*, 1995), which in turn stimulates the rapid production of $InsP_3$ and DAG (reviewed in Stojilkovic and Catt, 1992, 1995; Stojilkovic *et al.*, 1994). The production of $InsP_3$ stimulates several types of temporal $[Ca^{2+}]_i$ responses, including subthreshold, baseline spiking, biphasic nonoscillatory and biphasic oscillatory responses. Subthreshold responses occur in response to subphysiological concentrations of GnRH (≤ 10 pM) and are characterized by low amplitude, transient increases in

$[Ca^{2+}]_i$. Large amplitude, base-line spiking is evoked by physiological concentrations of GnRH (0.1 to 10 nM). In these responses, the frequency of Ca^{2+} spiking and the latency of the response, but not the amplitude, is dependent on the concentration of GnRH. In response to supraphysiological concentrations of GnRH (≥ 100 nM), biphasic non-oscillatory or biphasic oscillatory $[Ca^{2+}]_i$ responses are observed (Iida *et al.*, 1991; Stojilkovic *et al.*, 1993). In addition to these temporal differences, two spatial patterns of $[Ca^{2+}]_i$ responses to GnRH have been observed in rat gonadotrophs. The most common is a polarized wave. This is characterized by an initial increase in $[Ca^{2+}]_i$ at a localized point near the cell membrane from which a wave of $[Ca^{2+}]_i$ spreads throughout the cell. The second is characterized by a wave which originates from an evenly distributed increase in $[Ca^{2+}]_i$ around the cell border, termed a radial wave (Rawlings *et al.*, 1991).

In GnRH-stimulated rat gonadotrophs, oscillations in $[Ca^{2+}]_i$ occur without fluctuations in elevated $InsP_3$ levels (Stojilkovic *et al.*, 1993). This is consistent with second-messenger controlled models of Ca^{2+} signaling (Berridge, 1990). The $[Ca^{2+}]i$ response can be separated into two distinct phases, initiation and regeneration of $[Ca^{2+}]_i$ spiking (reviewed by Stojilkovic *et al.*, 1994). During GnRH receptor activation, an increase in $InsP_3$ formation initiates $[Ca^{2+}]_i$ spiking, the frequency of which is determined by the $InsP_3$ concentration. The increase in $InsP_3$ levels stimulates the release of Ca^{2+} from the endoplasmic reticulum. The initial rise in $[Ca^{2+}]_i$ potentiates $InsP_3$-receptor activity, further facilitating the release of Ca^{2+}. This results in a rapid release of $[Ca^{2+}]_i$. As $[Ca^{2+}]_i$ continues to elevate, it exerts an inhibition on Ca^{2+} release from $InsP_3$-sensitive stores, eventually terminating the $[Ca^{2+}]_i$ spike. This inhibition is subsequently released by a reduction in $[Ca^{2+}]_i$ due to the activity of the endoplasmic reticulum Ca^{2+}-ATPase, which pumps Ca^{2+} back into the agonist-sensitive Ca^{2+} stores. In the continual presence of $InsP_3$, another $[Ca^{2+}]_i$ spike can be initiated and repeated spiking can occur. An important requirement of this model is that the $[Ca^{2+}]_i$-induced inhibition of $InsP_3$ channels lasts long enough to allow Ca^{2+} to be pumped back to refill the agonist-sensitive stores. It has been suggested that Ca^{2+} passes through other intracellular compartments prior to returning to the $InsP_3$-sensitive store (Hille *et al.*, 1995). In response to sustained GnRH stimulation, this cycle will continue as long as the agonist-sensitive Ca^{2+} pool is not depleted.

To maintain the agonist-sensitive intracellular Ca^{2+} stores during prolonged GnRH stimulation, Ca^{2+} entry through voltage-dependent and -independent Ca^{2+} channels is essential. Extracellular Ca^{2+} entry is required to compensate for the small amount of Ca^{2+} lost from the agonist-sensitive pool due to its extrusion from the cell during each oscillatory rise in $[Ca^{2+}]_i$, as well as the sequestration of Ca^{2+} into agonist-insensitive stores. Entry of Ca^{2+} during GnRH stimulation is a consequence of rhythmic membrane potential activity mediated by the periodic opening and closing of apamin-sensitive, Ca^{2+}-activated K^+ channels (Kukuljan *et al.*, 1992; Tse and Hille, 1992). The periodic opening of these channels is due to the transient increases in $[Ca^{2+}]_i$ in response to GnRH-receptor activation (discussed above). This results in periodic membrane hyperpolarizations, which are believed to be important for the removal of voltage-dependent steady-state inactivation of Na^+ and Ca^{2+} channels (Tse and Hille, 1993). In response to a decrease in $[Ca^{2+}]_i$ during the falling phase of a $[Ca^{2+}]_i$ spike, the apamin-sensitive, Ca^{2+}-activated K^+ channel activity decreases. This leads to membrane depolarization and, once a threshold potential is reached, the firing of a few Na^+- and Ca^{2+}-dependent action potentials (Tse and Hille, 1992; Tse and Hille, 1993). As a result, Ca^{2+} enters the gonadotroph allowing, for the refilling of cytoplasmic Ca^{2+} stores. Thus, agonist induced $[Ca^{2+}]_i$ oscillations and ionic channels within the plasma membrane act in a coordinated fashion to regulate intracellular Ca^{2+} stores in rat gonadotrophs.

As previously mentioned, the primary function of GnRH-induced increases in intracellular $[Ca^{2+}]_i$ is the stimulation of hormone secretion. To directly evaluate the relationship between the acute $[Ca^{2+}]_i$ response to GnRH and hormone secretion in single gonadotrophs, high resolution recordings of cellular capacitance were used (Tse *et al.*, 1993). A sudden increase in plasma membrane capacitance indicates an increase in plasma membrane area and is assumed to represent an exocytoic event. Results from these studies demonstrate that GnRH-induced transient increases in $[Ca^{2+}]_i$ result in exocytosis. This response was not affected by removal of extracellular Ca^{2+} but was suppressed by buffering intracellular Ca^{2+}, indicating exocytosis is Ca^{2+} dependent but extracellular Ca^{2+} influx is not involved (Hille, *et al.*, 1994, 1995).

OBJECTIVES

The primary goal of this thesis is to investigate the actions of GnRH and dopamine on plasma membrane ionic currents and $[Ca^{2+}]_i$ in single identified goldfish gonadotrophs. Specifically, the hypothesis that GnRH and dopamine modulate the activity of voltage-dependent Ca^{2+} channels to alter $[Ca^{2+}]_i$ will be tested using a combination of electrophysiological, single-cell Ca^{2+} imaging and hormone-release experiments. However, it was first necessary to develop a method to identify goldfish gonadotrophs in mixed populations of dispersed goldfish pituitary cells. In experiments reported in Chapter 2, a method was developed to identify live goldfish gonadotrophs, as well as somatotrophs and lactotrophs, according to their unique cellular morphology when viewed under Nomarski differential interference-contrast microscopy. This method allowed for subsequent electrophysiological and single-cell Ca^{2+}-imaging experiments on a subpopulation of goldfish gonadotrophs

Following the development of the cell identification technique, the electrical membrane properties and ionic currents present in goldfish gonadotrophs were characterized using whole-cell, patch-clamp recording techniques (Chapter 3). These studies, the first of their kind in fish gonadotrophs, revealed voltage dependent Na^+, Ca^{2+} and K^+ currents in goldfish gonadotrophs. However, unlike mammalian gonadotrophs, Ca^{2+}-activated K^+ currents were not present in goldfish gonadotrophs. These studies provide the foundation for further investigation of the electrical membrane events involved in GnRH and dopamine regulation of GTH-II secretion.

The presence of voltage-dependent Na^+ currents in goldfish gonadotrophs prompted the investigation of the involvement of extracellular Na^+ in mediating GnRH action in goldfish gonadotrophs (Chapter 4). Although extracellular Na^+ regulates a diverse array of cellular functions in many different cell types, its involvement in the regulation of GTH secretion in vertebrates has received little attention and remains controversial at best. The physiological role of extracellular Na^+ was investigated using a combination of hormone release studies under static incubation and perifusion conditions, and whole-cell patch-clamp recording experiments. The data in this chapter clearly demonstrate, for the first time in vertebrate gonadotrophs the involvement of Na^+/H^+ antiports in GnRH action under physiological conditions.

In Chapter 5, the effects of dopamine on voltage-dependent Na^+, Ca^{2+} and K^+ currents were investigated using a combination of hormone release and patch-clamp techniques. The results from this study directly demonstrate the inhibitory effects of dopamine D_2 receptor activation on voltage-dependent Ca^{2+} currents in goldfish gonadotrophs.

The actions of GnRH and one of its known second messengers, PKC, in the regulation of GTH-II secretion were investigated using hormone release, patch-clamp and Ca^{2+} imaging techniques in Chapter 6 and 7. Chapter 6 describes the development of a single-cell fura-II Ca^{2+}-imaging technique for the measurement of intracellular Ca^{2+} dynamics in identified goldfish gonadotrophs and describes the effects of GnRH on these parameters. The effects of PKC activation were reported in Chapter 7. Results presented in these chapters provide preliminary data on agonist-induced increases in $[Ca^{2+}]_i$ in goldfish gonadotrophs. In addition, these data extend our knowledge on the spatial aspects of changes in $[Ca^{2+}]$i following agonist stimulation in vertebrate gonadotrophs in general, an area which has received little attention compared to the temporal aspects of $[Ca^{2+}]$i alterations.

Finally, the last chapter integrates the findings in this thesis into the existing model of GnRH action in goldfish gonadotrophs. New insights of how GnRH and dopamine interact to stimulate the rapid increase in circulating GTH-II required to induce ovulation in goldfish are also discussed.

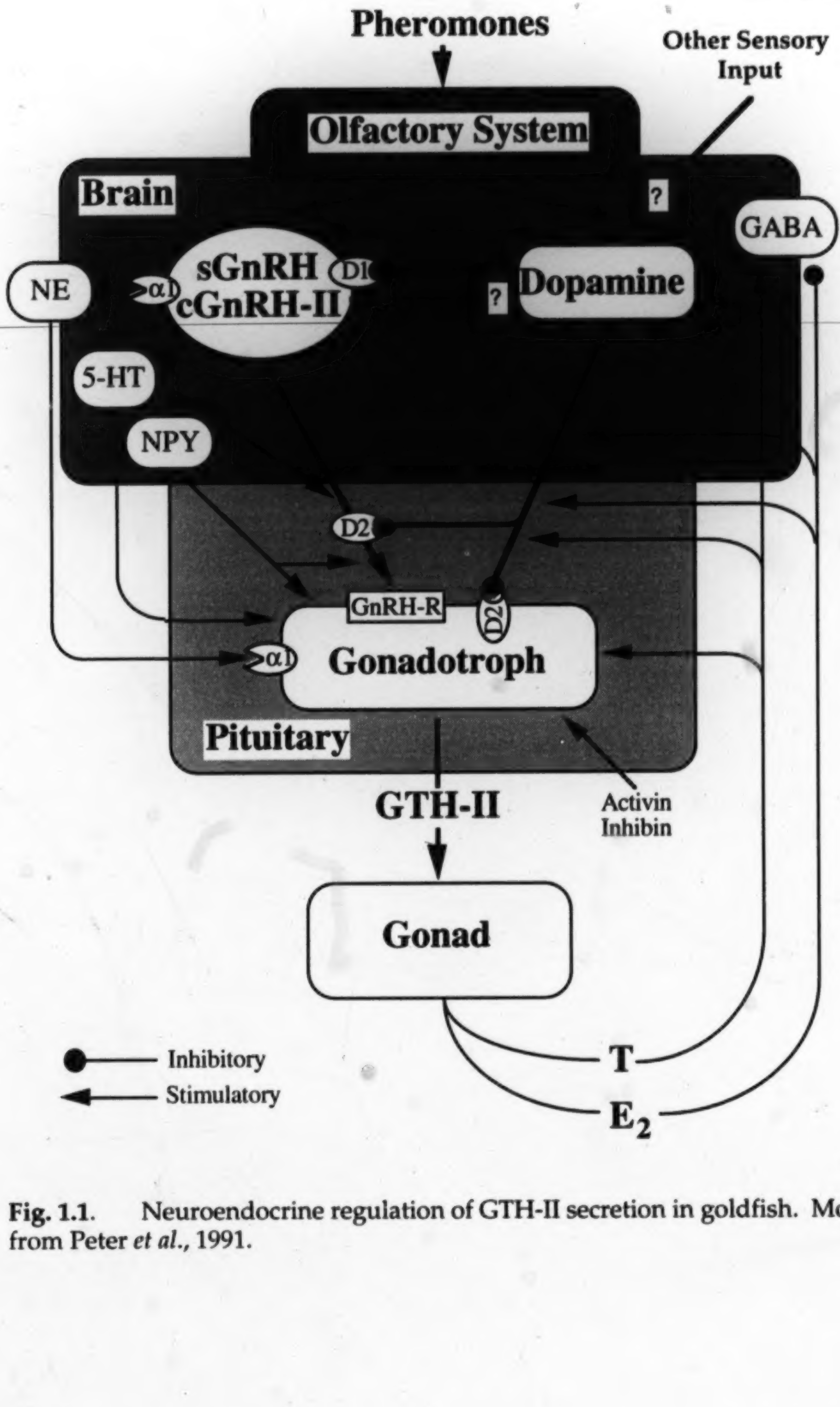

Fig. 1.1. Neuroendocrine regulation of GTH-II secretion in goldfish. Modified from Peter *et al.*, 1991.

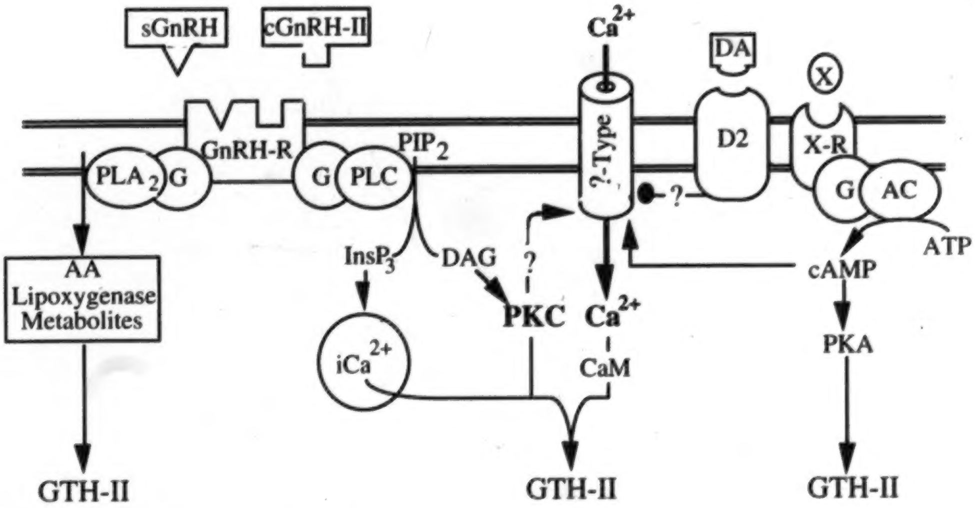

Fig. 1.2. Signal transduction pathways in goldfish gonadotrophs. See text for abbreviations. The goal of this thesis was to test the effects of the signal transduction pathways indicated by a (?) on the regulation of voltage-dependent Ca^{2+} channels.

Chapter 2

MORPHOLOGICAL IDENTIFICATION OF LIVE GONADOTROPHS, SOMATOTROPHS, AND LACTOTROPHS IN GOLDFISH PITUITARY-CELL CULTURES[†]

INTRODUCTION

The neuroendocrine factors and intracellular signaling mechanisms mediating GTH-II and GH secretion in goldfish have been studied *in vivo* and *in vitro* using pituitary fragments and mixed populations of dispersed pituitary cells (Peter *et al.*, 1986, 1990a, 1990b; Chang *et al.*, 1993). However, to further understand the mechanisms underlying pituitary-hormone secretion, it will be necessary to study the physiology of identified single cells.

In goldfish, GTH-II secretion is under the stimulatory influence of the two endogenous GnRHs, sGnRH and cGnRH-II (Peter *et al.*, 1990b). It has been demonstrated that both GnRHs compete for the same class of high-affinity, low-capacity receptors on gonadotrophs to elicit GTH-II release (Habibi *et al.*, 1987; Cook *et al.*, 1991). However, recent evidence suggests that the two endogenous GnRHs stimulate GTH-II secretion via partially different intracellular signaling pathways (Chang *et al.*, 1993; Chang *et al.*, 1996). These results raise the possibility that discrete subpopulations of gonadotrophs respond differently to the native GnRHs. The ability to identify individual gonadotrophs is essential for testing this hypothesis.

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In addition to stimulating GTH-II release, salmon GnRH and chicken GnRH-II stimulate GH release in goldfish (Peter *et al.*, 1990a). Initial reports indicate that the signal transduction pathways involved in GnRH-stimulated GTH-II and GH release are different (Chang *et al.*, 1989; Chang and de Leeuw, 1990). Identification of gonadotrophs and somatotrophs will help to elucidate the differences between the signal-transduction pathways involved in GnRH-stimulated GTH-II and GH release.

The pituitary gland in all vertebrates is made up of several different hormone-secreting cell types. Consequently, to facilitate the study of distinct pituitary-cell types, several cell-enrichment methods have been developed. These include unit-gravity sedimentation, density-gradient centrifugation, centrifugal elutriation, and fluorescence-activated cell sorting (Childs *et al.*, 1983; Hymer and Hatfield, 1984). Although cell enrichment provides an opportunity to study individual hormone-secreting cell types with a relatively high degree of confidence, it partially negates the paracrine interactions between different cell types. The importance of paracrine interactions in the regulation of anterior pituitary-cell function has been previously demonstrated (Denef and Andries, 1983). Positive cell identification in enriched and in mixed cell populations has also been achieved by reverse hemolytic plaque assay (Luque *et al.*, 1986; Tse and Hille, 1992). This method provides an opportunity to study distinct hormone-secreting cell types within heterogeneous populations of pituitary cells; however, unless the assay is performed subsequent to physiological studies, normal cell function may be compromised by the procedure. For example, sheep pars distalis gonadotrophs identified by plaque assay showed inconsistent bioelectrical activity and responses to GnRH compared to gonadotrophs from enriched populations (Heyward *et al.*, 1993). Therefore, for the purpose of physiological studies of identified pituitary-cell types within mixed cell cultures, the development of an alternative identification procedure is warranted.

In the present chapter, a method was developed to directly identify goldfish gonadotrophs, somatotrophs and lactotrophs in mixed, as well as in enriched pituitary-cell populations, by their unique cellular morphologies. This method of cell identification was used for all subsequent single-cell electrophysiological and single-cell Ca^{2+} imaging studies reported in this thesis.

MATERIALS AND METHODS

Fish maintenance and cell dispersion

Common goldfish (8-13 cm in body length) were purchased from Grassyforks Fisheries (Martinsville, IN, USA) and Ozark Fisheries (Stoutland, MO, USA) and held in flow-through aquaria (1800 l). Fish were maintained at 17-20°C on a simulated natural photoperiod (adjusted weekly according to the times of sunset and sunrise in Edmonton, AB, Canada) and fed to satiation with commercial fish food daily. Fish were acclimatized to the above conditions for at least seven days prior to use. Sexually matured (prespawning), recrudescent and regressed male and female fish were used.

Fish were anesthetized in 0.05% tricaine methanesulfonate prior to decapitation. Pituitaries were removed and placed in dispersion medium (Medium 199 with Hanks' salts, 25 mM HEPES, 2.2 g/l sodium bicarbonate, 0.3% bovine serum albumin (BSA), 100 000 U/l penicillin, and 100 mg/l streptomycin, pH 7.2; Gibco, Grand Island, NY, USA). Pituitary cells were dispersed using the trypsin/DNAse treatment procedure described by Chang *et al*, (1990a).

Cell separation

Dispersed pituitary cells suspended in Ca^{2+}-free medium (M199 with Hanks' salts, prepared without $CaCl_2$ and containing 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100 000 U/l penicillin, 100 mg/l streptomycin and 0.3% BSA, pH 7.2; Gibco) were separated using a discontinuous Percoll density-gradient cell-separation method as described in Chang and Jobin (1994b). Briefly, a stock solution of Percoll (Pharmacia, Piscataway, NJ, USA) was prepared in 10X Dulbeco's Ca^{2+}-free phosphate-buffered saline (10 X D-PBS; 2g/l KCl, 2 g/l KH_2PO_4, 80 g/l NaCl, 21.6 g/l $Na_2HPO_4 \bullet 7H_2O$) with 5% BSA (9:1; Percoll:10X D-PBS) and diluted with 1X D-PBS with 0.5% BSA to 40, 50, 60, 70, and 80% Percoll. A discontinuous gradient was built up using 8 ml of each density suspension in a 50-ml disposable centrifuge tube. Dispersed pituitary cells were gently loaded on top of the gradient and centrifuged at 1400 x g for 25 min at 20°C using a horizontal rotor. After centrifugation, six cell bands were visible, one at each of the step-gradient interphases and one at the bottom of the tube. These fractions are referred to as fractions 1-6, respectively, with fraction 1 being at the 0%/40% Percoll junction. The cells from each band were carefully collected, washed with 10 ml Ca^{2+}-free medium, and harvested by centrifugation at 900 x g for 10 min at 20°C. Harvested cells were resuspended in 2.5 ml Ca^{2+}-free medium, and the cell yield and viability in each fraction were calculated by counting in a hemocytometer in the presence of trypan blue. Aside from fraction 1, which had an average cell viability of 67.7 ± 6.4% (mean ± SEM; $n = 6$), all other fractions contained cells that were 83–95% viable as determined by the trypan blue-exclusion test (Chang *et al.*, 1990a). The cell fractions were also tested for their ability to release GTH-II and GH using perifusion studies (Chang *et al.*, 1990b).

Cellular hormone-content analysis

To measure hormone content, aliquots of cells from each fraction were placed in 1 ml distilled and deionized water, frozen, and then thawed to release cell contents. The samples were stored at -20°C until hormone content could be measured by radioimmunoassay (RIA). For GTH-II and GH, cellular contents (ng hormone/10^6 cells) in each of the Percoll fractions were normalized as a percentage of the value obtained for fraction 1. The data from all experiments were pooled. Statistical analysis of cell content for the pooled data was performed by analysis of variance (ANOVA) followed by Fisher's least significance difference test (LSD). Differences were considered significant when $P < 0.05$.

Radioimmunoassay

GH levels from both bioactivity studies and cell-content analysis were measured using a RIA for carp GH, previously validated for use in the goldfish by Marchant *et al.*, (1989). For GTH-II, an RIA specific for maturational GTH-II was used to measure GTH-II levels from perifusion studies and cell-content analysis (Peter *et at.*, 1984; Van Der Kraak *et al.*, 1992). Samples from each experiment were measured in duplicate in the same RIA.

Cell-culture immunocytochemistry

Dispersed pituitary cells were cultured overnight in poly-L-lysine-coated (0.01 mg/ml) glass-bottom petri dishes containing culture medium (M199 with Earle's salts, 1% horse serum, 25 mM HEPES, 2.2 g/l sodium bicarbonate, 100 000 U/l penicillin, and 100 mg/l streptomycin, pH 7.2; Gibco). Dispersed cells were fixed in Zamboni's 2X fixative (4% paraformaldehyde and 7.5% saturated picric acid in phosphate buffer [20 g/l $NaH_2PO_4 \bullet H_2O$, 32.5 g/l Na_2HPO_4]; pH 7.3) for 90 min. After fixation, the cells were rinsed repeatedly with 1X D-PBS for 60 min. Cells were preincubated in 1X D-PBS containing 0.1% Triton X-100 (Sigma Chemical Company, St. Louis, MO, USA) and 10% horse serum (Gibco) for 30 min, prior to primary antibody treatment. Cells for immunocytochemistry were processed with the same antibodies used in the RIA, but at different concentrations. Cells were incubated in primary antisera (rabbit anti-carp-GH at 1:3000, anti-carp-GTH-II at 1:6000, and anti-carp-PRL at 1:3000) diluted in 1X D-PBS with 0.2% horse serum for 12 hr. The specificity of these primary antiserum concentrations has been previously demonstrated (Cook *et al.*, 1991; Van Der Kraak *et al.*, 1992). Cells were washed repeatedly with D-PBS containing 0.01% Triton X-100 for a total of 30 min and then incubated in goat anti-rabbit IgG conjugated to fluorescein (Sigma) diluted 1:200 in D-PBS with 0.01% Triton X-100 for 60 min. Following this incubation, cells were washed five times with D-PBS containing 0.01% Triton X-100 for a total of 30 min and then two times with D-PBS before mounting in glycerol. All treatments were performed at room temperature with the exception of the fixation and primary antiserum incubations, which were performed at 4°C. Cells were viewed and photographed using a Nikon Diaphot TMD inverted or a Nikon Microphot FXA upright microscope, both equipped with epifluorescence illumination and Nomarski differential interference-contrast (DIC) optics. Differences in percentage of cells staining between fractions were analyzed by ANOVA followed by Fisher's least significance difference test. Differences were considered significant when $P < 0.05$.

Reliability of cell-type identification in immunofluorescently labeled cell preparations

For each cell type, an independent observer was shown photomicrographs of a particular cell and given a list of the morphological characteristics describing the cell. Using DIC microscopy, the observer was then asked to select individual cells, in either mixed cell populations or specific enriched cell fractions, which displayed the characteristic morphologies of the cell type in question. A second independent observer was then asked to view the cell under epifluorecence illumination and record if the selected cell stained positive for the cell type in question. Due to the low GTH-II, GH, and PRL cell content in fractions 1, 2, and 6, only fractions 3–5 were used for this part of the study.

Identification of gonadotrophs, somatotrophs, and lactotrophs in mixed populations of live pituitary cells

The identities of live gonadotrophs, somatotrophs and lactotrophs were predicted in mixed populations of dispersed pituitary-cell preparations using their morphological characteristics as previously established for immunofluorescently-treated pituitary cell types. The locations of the selected cells were marked, and the identity of each cell was verified following processing for immunofluorescence microscopy using GTH-II, GH, and PRL antisera, as described above. A grid was drawn on the underside of a glass-bottom petri dish prior to cell culture. Under DIC microscopy, the location of the field containing the selected cells was marked relative to the grid pattern, and the distribution of the cells in each individual field was carefully sketched. Following processing for immunocytochemistry, selected cells were relocated using the grid and cell-map sketches.

RESULTS

Cell separation

To aid in the initial characterization of the individual cell types, a discontinuous Percoll gradient cell-separation technique was used to obtain fractions enriched with gonadotrophs, somatotrophs and lactotrophs. After cell separation, 83.1 ± 4.4% (*n* = 6) of the total cells loaded were recovered in the six cell fractions. The average percentage of total viable cells recovered for each fraction is given in Table 2.1. Approximately 75% of the total cells recovered were in fractions 2 and 3. Fraction 6 contained negligible amounts of cells and, for this reason, was not used in the subsequent experiments. Immunofluorescent staining and cell-content analysis were used to determine which fractions were enriched with gonadotrophs, somatotrophs and lactotrophs compared to unfractionated dispersed pituitary-cell populations. The total number of gonadotrophs, somatotrophs and lactotrophs in unfractionated as well as fractionated populations of dispersed pituitary-cell preparations was determined by immunocytochemistry. In the present study, background staining was minimal compared to positively stained cells (for example, see Fig. 2.1), allowing for the specific identification of cell types. In unfractionated pituitary-cell culture, 15% of the cells stained for GTH-II, 20% stained for GH, and 15% stained for PRL. Compared to unfractionated dispersed pituitary-cell preparations, fraction 5 contained a significantly greater percentage of gonadotrophs (Fig. 2.2). These results were similar to those obtained in preliminary experiments in which GTH-II-containing cells were immunolabelled using peroxidase-staining techniques (J. P. Chang and O. Kah, unpublished data). In contrast, fractions 3 and 4 contained greater percentages of lactotrophs and somatotrophs, respectively (Fig. 2.2). Similarly, cellular hormonal contents for GTH-II and GH were greatest in fractions 5 and 4, respectively (Fig. 2.3). Therefore, based on immunocytochemistry and cell-content analysis, it was determined that enriched gonadotroph, somatotroph and lactotroph populations were found in fractions 5, 4, and 3, respectively. Furthermore, hormone-release studies using a cell column perifusion and a static incubation system (Chang *et al.*, 1990a, 1990b) confirmed that these fractionated cell populations responded normally to neuroendocrine regulators of GTH-II and GH secretion, such as GnRH and dopamine (Peter *et al.*, 1986, 1990b; Wong *et al.*, 1992). The GTH-II response to GnRH was markedly increased in enriched gonadotroph populations compared to that from mixed populations of dispersed pituitary cells (Jobin, 1993). Similarly, the GH response to dopamine was increased by sevenfold in enriched somatotroph populations (Chang and Wong, unpublished results).

Morphology of gonadotrophs, somatotrophs and lactotrophs

Gonadotrophs. The morphology of immunofluorescently labelled gonadotrophs was characterized by DIC microscopy (Fig. 2.4). These cells were usually ovoid and were the largest of the three cell types examined (15.6 ± 0.7 μm, n = 12; range = 12.0 to 19.0 μm). Their most prominent feature was the presence of a small reniform nucleus which occupied less than one-third of the cross-sectional area of the cell. The nucleus was always located at the cell periphery, with its concave surface facing centrally. Using the presence of this small reniform nucleus as a reliable indicator of gonadotroph type (see below), the morphology of live gonadotrophs in mixed pituitary-cell culture was also characterized. Live gonadotrophs were of similar size (16.0 ± 0.4 µm, n = 20; range = 13.0 to 19.0 µm) and shape as immunofluorescently labelled cells. The reniform nucleus was equally prominent in both live and labelled cells. Moreover, a single, large, irregularly shaped nucleolus could usually be detected in these cells. The cytoplasm of live gonadotrophs was characterized by the presence of numerous small granules, large spherical globules, and a few larger spherical or irregularly shaped inclusions. Cells which exhibited all of these features were morphologically distinct from the other cell types and reliably stained with GTH-II anti-serum (see below).

Somatotrophs. The morphology of immunofluorescently labelled somatotrophs was characterized by DIC microscopy (Fig. 2.5). Immunofluorescently labelled somatotrophs were spherical and measured 10.4 ± 0.2 µm in diameter (n = 14; range = 9.0 to 11.0 µm). The most distinctive feature of these cells was a large ovoid nucleus containing a central nucleolus. The nucleus occupied approximately half of the cross-sectional area of the cell and was usually eccentric in position. The features of labelled cells were also characteristic of live cells. In addition, live somatotrophs had several small cytoplasmic extensions. The cytoplasm of somatotrophs was characterized by numerous small granules, however unlike gonadotrophs, large globules were never observed. Cells exhibiting all of these features were easily distinguishable from other cell types and reliably stained with GH anti-serum (see below).

Lactotrophs. The morphology of immunofluorescently labelled lactotrophs was characterized by DIC microscopy (Fig. 2.6). Immunofluorescently labelled lactotrophs were spherical cells, measuring

10.5 ± 0.2 µm in diameter ($n = 8$; range = 10.0 to 11.0 µm). Their cytoplasm had a rough granulated appearance and contained a large central nucleus. The nucleus was irregularly shaped, occupied more than half of the cross-sectional area of the cell, and had no apparent nucleolus. The same morphological characteristics were observed in live lactotrophs and were a reliable indicator of this cell type (see below).

Reliability of cell-type identification in immunofluorescently labelled and live cell preparations

To determine the reliability of the identification protocol developed for immunofluorescently labelled gonadotrophs, somatotrophs, and lactotrophs, an independent observer was asked to view cells under DIC optics and identify each cell type according to the characteristics described in the previous section. A second observer, using fluorescence optics, then scored whether the cells contained the predicted immunoreactivity. Among the three cell types, gonadodotrophs and lactotrophs could be easily distinguished from one another and from other cell types. Gonadotrophs and lactotrophs were correctly identified in more than 90% of the trials in fractions 3–5, as well as in unfractionated cell populations (Table 2.2). The accuracy of somatotroph identification was slightly reduced in fraction 3 and in unfractionated cell populations. However, somatotrophs were correctly identified in 90% of the trials in the GH-enriched fraction 4 (Table 2.2).

Since the primary objective of this study was to identify pituitary-cell types in live, dispersed cell cultures, the reliability of the identification protocols was also examined using live cells. Cells were identified according to morphological characteristics, with subsequent confirmation of cell identities by immunofluorescence. Gonadotrophs were correctly identified in 92% (n =

36) of the trials in live unfractionated pituitary-cell cultures. Similarly, somatotrophs and lactotrophs were correctly identified in 94% (*n* = 32) and 100% (*n* = 8) of the trials, respectively (Table 2.2).

Lack of colocalization of GTH-II, GH and PRL in identified cells

In fractions 4 and 5, the combined percentages of gonadotrophs, somatotrophs and lactotrophs present within the cell population were 105 and 102%, respectively. This gave rise to the possibility that GTH-II, GH or PRL may be colocalized within single cells. To examine this possibility, we tested whether cells displaying a morphology characteristic of one cell type were positively stained for another cell type (Table 2.3). When gonadotrophs and lactotrophs were identified by their unique morphological characteristics as described above, no evidence for co-localization was found. In contrast, 4% of the predicted somatotrophs stained for GTH-II or PRL. Thus, colocalization of GTH-II, GH or PRL within single cells was not commonly observed in the particular cell types that were morphologically characterized in this study.

DISCUSSION

This study presents a method for the identification of gonadotrophs, somatotrophs, and lactotrophs in unfractionated and enriched primary cell cultures. Gonadotrophs, somatotrophs, and lactotrophs, when viewed by DIC microscopy, exhibit morphologies which are specific for the cell type. By combining DIC microscopy with immunofluorescence, we demonstrated that the characteristic morphology of gonadotrophs, somatotrophs, and lactotrophs is a sufficient and reliable indicator of cell identity. The ability to morphologically identify gonadotrophs, somatotrophs, and lactotrophs in either unfractionated or enriched primary cell culture has several advantages over previous methods of pituitary-cell identification. Firstly, while cell-separation techniques and immunological assays are effective methods of identification, all involve complex treatments which may alter cell function. In contrast, morphological identification of single cell types in culture allows the immediate and direct study of a known cell type without further modification of its surrounding environment or itself. Secondly, by combining the ability to directly identify cell types in culture with cell-enrichment techniques, it will be possible to study the paracrine interactions between cell types. Thirdly, preparation of enriched cell cultures often involves cell loss (average loss = 17%, range = 6 to 36%, in the present study). The ability to identify single cell types in primary cultures of unfractionated cell populations represents a potential saving on experimental animal and material costs. Therefore, morphological identification of pituitary-cell types provides a simple and economical means to carry out single-cell analysis of pituitary cells under various physiological conditions.

Using a discontinuous Percoll gradient cell-separation technique, we were able to obtain fractions enriched with gonadotrophs, somatotrophs, and lactotrophs. Although fractions enriched with gonadotrophs, somatotrophs, and lactotrophs were contaminated with other pituitary-cell types, these enriched fractions facilitated the initial morphological characterization of the three cell types. Gonadotrophs were the largest of the cell types examined. They were ovoid in shape and contained a small reniform nucleus, small granules, large spherical globules, and irregularly shaped cytoplasmic inclusions. In previous light-microscope studies, the presence of large globular inclusions was shown to be a morphological characteristic of gonadotrophs in goldfish and other teleosts (Sage and Bern, 1971; Nagahama, 1973; Kaul and Vollrath, 1974). Irregularly shaped cytoplasmic inclusions may be the equivalent of the large vacuoles observed in gonadotrophs during certain stages of the reproductive cycle (Sage and Bern, 1971; Nagahama, 1973). The identity and function of the structures observed in the present study remain to be confirmed. Nevertheless, these cytoplasmic structures, together with the small reniform nucleus, gave gonadotrophs a clearly distinguishable and unique cellular morphology.

In teleost fishes, somatotrophs are ovoid acidophilic cells, measuring 8 to 13 µm in diameter, located in the proximal pars distalis (Sage and Bern, 1971; Kaul and Vollrath, 1974; Nagahama, 1974). The somatotrophs identified in the present study were similar in size and shape to those described earlier. Also consistent with previous light-microscope studies (Sage and Bern, 1971; Kaul and Vollrath, 1974), these cells contained large ovoid nuclei which were usually located eccentrically. In addition, a granulated cytoplasm and cytoplasmic extensions were also easily discernible by DIC microscopy. These features, taken together served as reliable markers of somatotrophs.

Lactotrophs in freshwater teleosts have been reported to range from 7 to 15 µm in diameter and to contain a central, prominent nucleus (Nagahama, 1973). In the present study, goldfish lactotrophs were of similar size and also possessed a large, irregularly shaped, central nucleus.

Male and female goldfish from all stages of the seasonal gonadal cycle were used in the present study. Seasonal variations in GTH-II and GH release have been reported in the goldfish. The number of high-affinity GnRH receptors and the sensitivity of the GTH-II response to GnRH stimulation varies seasonally (Sokolowska *et al.*, 1985a; Habibi *et al.*, 1989). The relative number of gonadotrophs and the amount of secretory granules within cells also increase with the onset of gonadal development (Nagahama, 1973).

Serum-GH levels in goldfish have also been shown to vary throughout the year; these seasonal changes in serum-GH levels may be partially related to the amounts of somatostatin present in the neuroendocrine regulatory centers (Marchant *et al.*, 1989). Irrespective of these possible qualitative and quantitative seasonal changes in goldfish anterior pituitary-cell function, gonadotrophs, somatotrophs, and lactotrophs exhibiting the characteristics described in the present study were always present in cell preparations obtained from fish at different stages of the gonadal cycle. However, it should be pointed out that cells identified by the morphological characteristics represent only a subpopulation of their respective cell types.

The results presented here demonstrate for the first time that distinct hormone-secreting pituitary-cell types can be directly identified in mixed and enriched cell populations with minimal pretreatment. Cell identification by this method can be used to examine the physiological properties of single identified cells using electrophysiological, calcium imaging, and other diagnostic techniques. Also, in combination with cell-enrichment techniques, the present method for pituitary-cell identification allows for physiological studies of single identified cells in controlled (select) environments of paracrine or intercellular interactions. Results from these experiments will greatly enhance our current knowledge of the regulation of pituitary-hormone release. The technique described here for the direct identification of discrete subpopulations of gonadotrophs, somatotrophs, and lactotrophs, based on the unique morphological differences, has a wide range of potential uses and may be adapted for use with other cell-culture preparations.

Table 2.1. Distribution of dispersed pituitary cells following separation with a discontinuous Percoll density gradient.

Fraction	Percentage of total cells recovered mean ± SEM (n = 6)
1	9.0 ± 1.8
2	40.3 ± 5.0
3	35.4 ± 4.4
4	15.6 ± 2.8
5	3.7 ± 1.7
6	0.4 ± 0.1

Table 2.2 Positive identification of gonadotrophs, somatotrophs, and lactotrophs in unfractionated and fractionated cell populations.

	Percentage of cells correctly identified (*n*)		
Fraction	Gonadotrophs	Somatotrophs	Lactotrophs
Immunofluorescently-labelled cells			
Unfractionated	100.0 (15)	80.0 (10)	97.0 (33)
3	95.7 (23)	75.0 (28)	100.0 (44)
4	90.0 (20)	90.2 (41)	100.0 (23)
5	100.0 (19)	ND	100.0 (10)
Total	96.4 (77)	81.7 (79)	99.3 (110)
Live primary cell culture			
Unfractionated	92.0 (36)	94.0 (32)	100.0 (8)

ND = not determined.

Table 2.3 Lack of evidence for GTH-II, GH or PRL co-localization within identified cell types.

	Percentage of identified cells stained with antiserum (as) specific for other hormone. ( ) = *n*		
Predicted cell type	GTH-II-as	GH-as	PRL-as
Gonadotrophs	----------	0.00 (40)	0.00 (50)
Somatotrophs	4.20 (70)	----------	4.62 (65)
Lactotrophs	0.00 (62)	0.00 (50)	----------

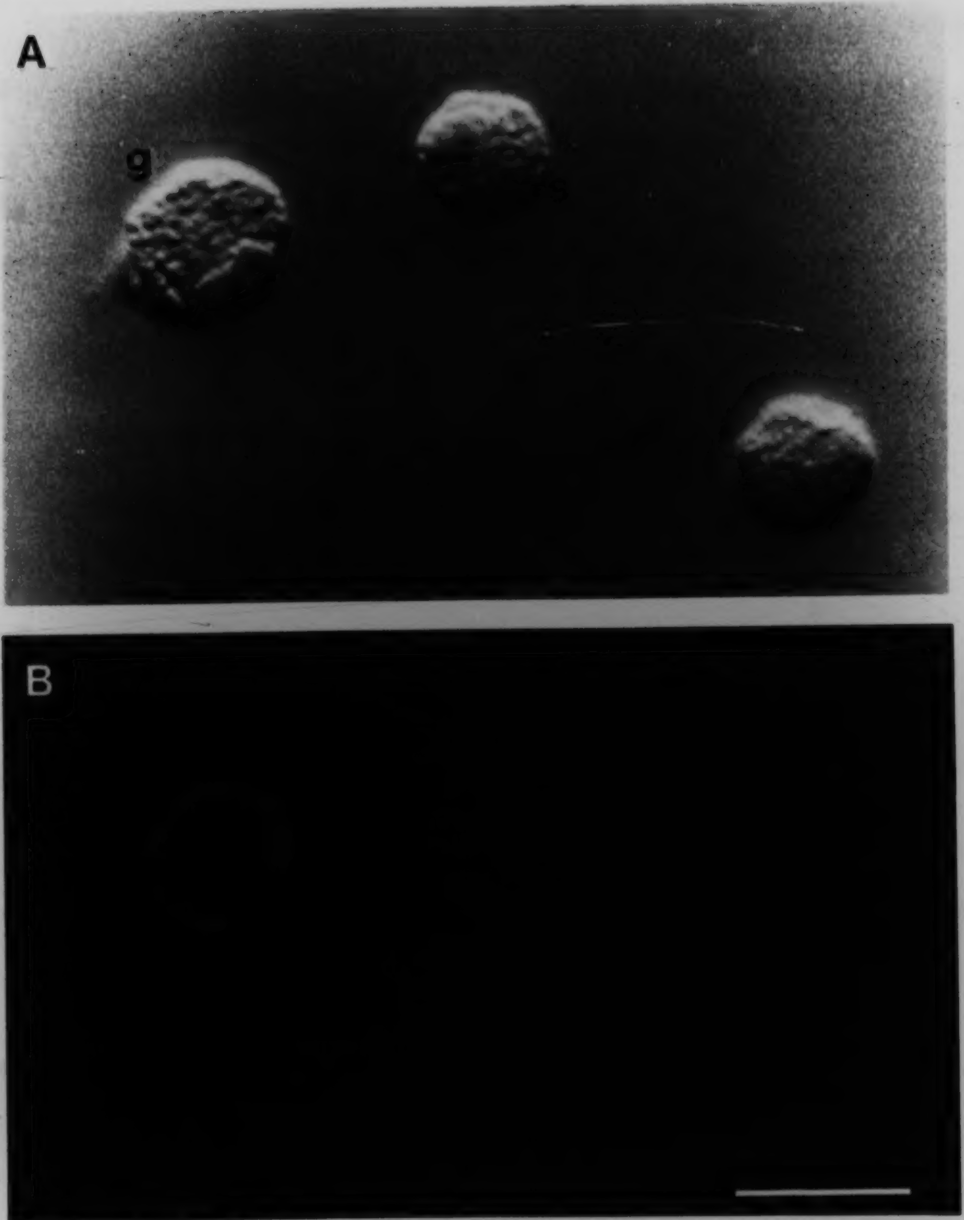


Fig. 2.1. Specificity of staining for gonadotrophs in mixed cell culture. A DIC photomicrograph of three cells with gonadotroph-like (*g*), somatotroph-like (*s*), and unidentified morphologies. Note the characteristic reniform nucleus in the gonadotroph-like cell. B Fluorescent photomicrograph of the identical field as in A. Only the cell with gonadotroph-like morphology was labelled by the anti-GTH-II antiserum. x1470 *Bar*: 15 µm

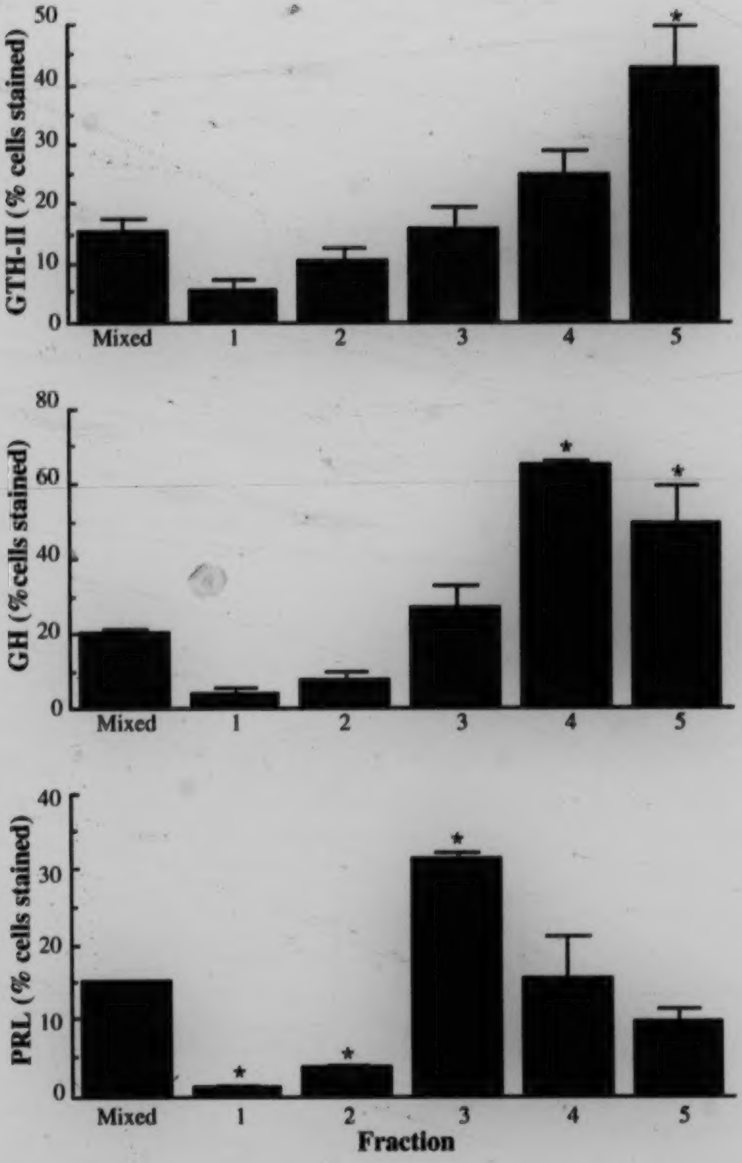


Fig. 2.2. Distribution of gonadotrophs, somatotrophs, and lactotrophs in fractionated dispersed cell populations as demonstrated by immunofluorescent staining. Pooled results (mean ± SEM) from four replicate experiments are presented. *Asterisks* indicate significant differences from unfractionated cell populations.

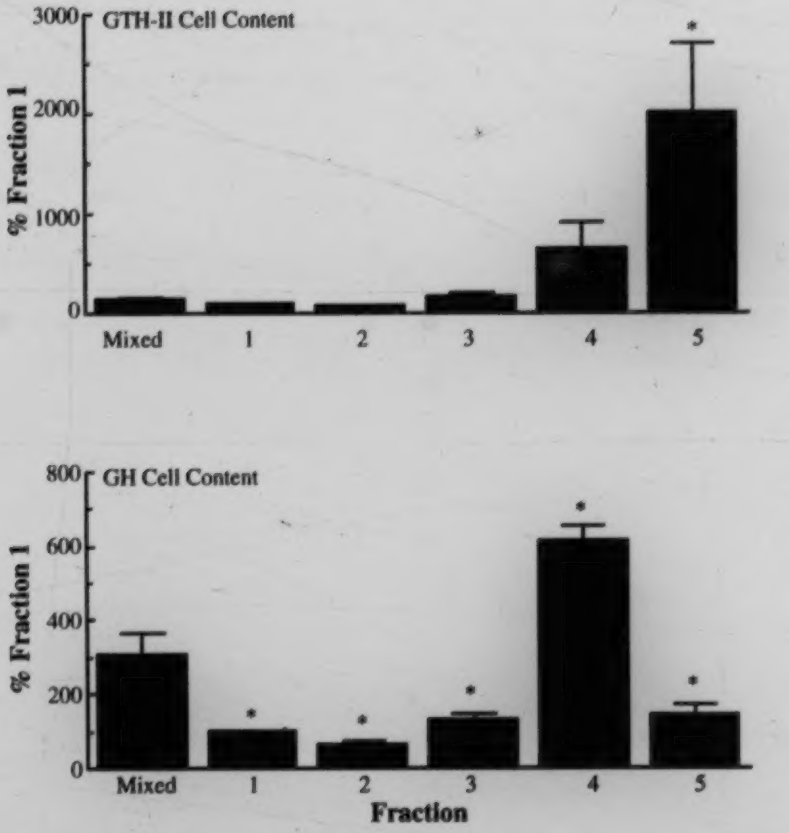


Fig. 2.3. GTH-II and GH cell content in fractionated and unfractionated cell populations. Pooled results (mean ± SEM) from six replicate experiments are presented. GTH-II and GH cell content (ng hormone/10^6 cells) were normalized as a percentage of the value obtained in fraction 1. *Asterisks* indicate significant differences from unfractionated cell populations.

Fig. 2.4. Morphology of gonadotrophs as revealed by DIC microscopy. The reniform nucleus (*N*) is readily apparent in immunofluorescently labelled (A) and live (B, C) gonadotrophs. Large spherical globules (*arrows*) and irregularly shaped inclusions (*arrowhead*) are also characteristic of this cell type. x3600. *Bar*: 5 µm

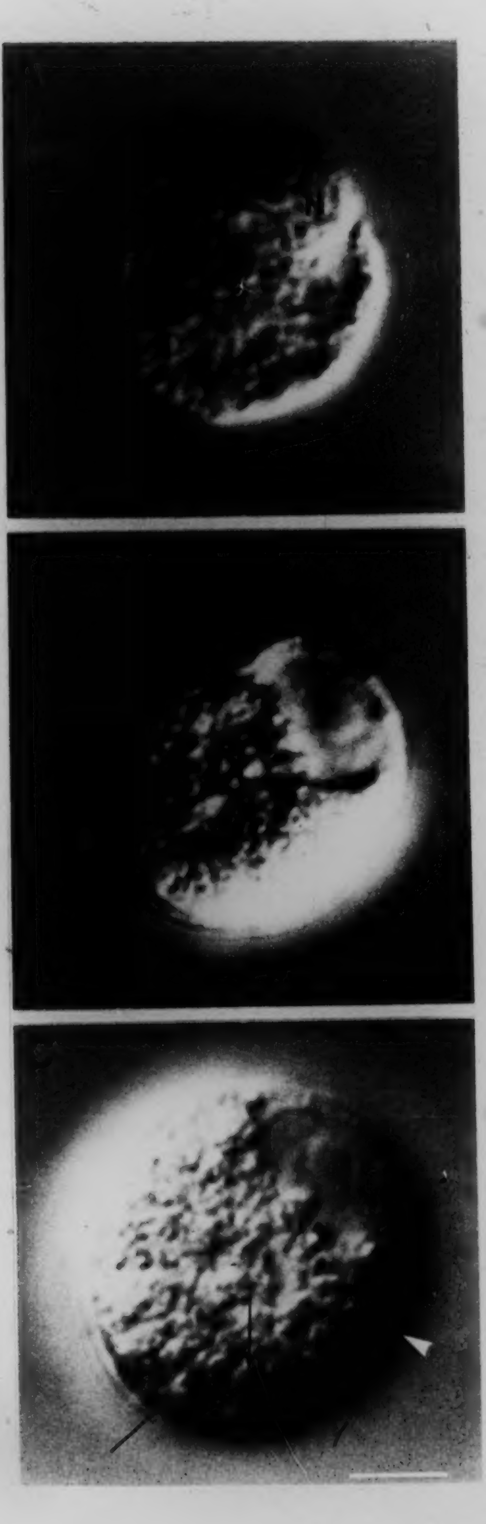


Fig. 2.5. Morphology of somatotrophs as revealed by DIC microscopy. The large ovoid nucleus is readily apparent in immunofluorescently labelled (A) and live (B, C) somatotrophs. Cytoplasmic extensions (*arrow heads*) are also characteristic of live GH cells in culture. x 3800. Bar: 5 μm

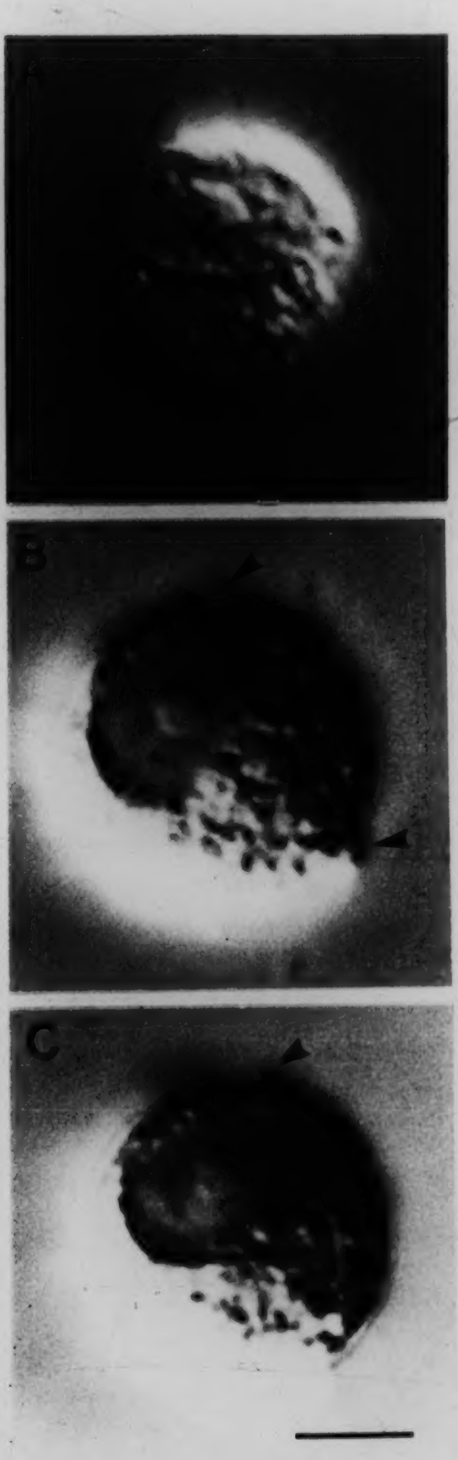
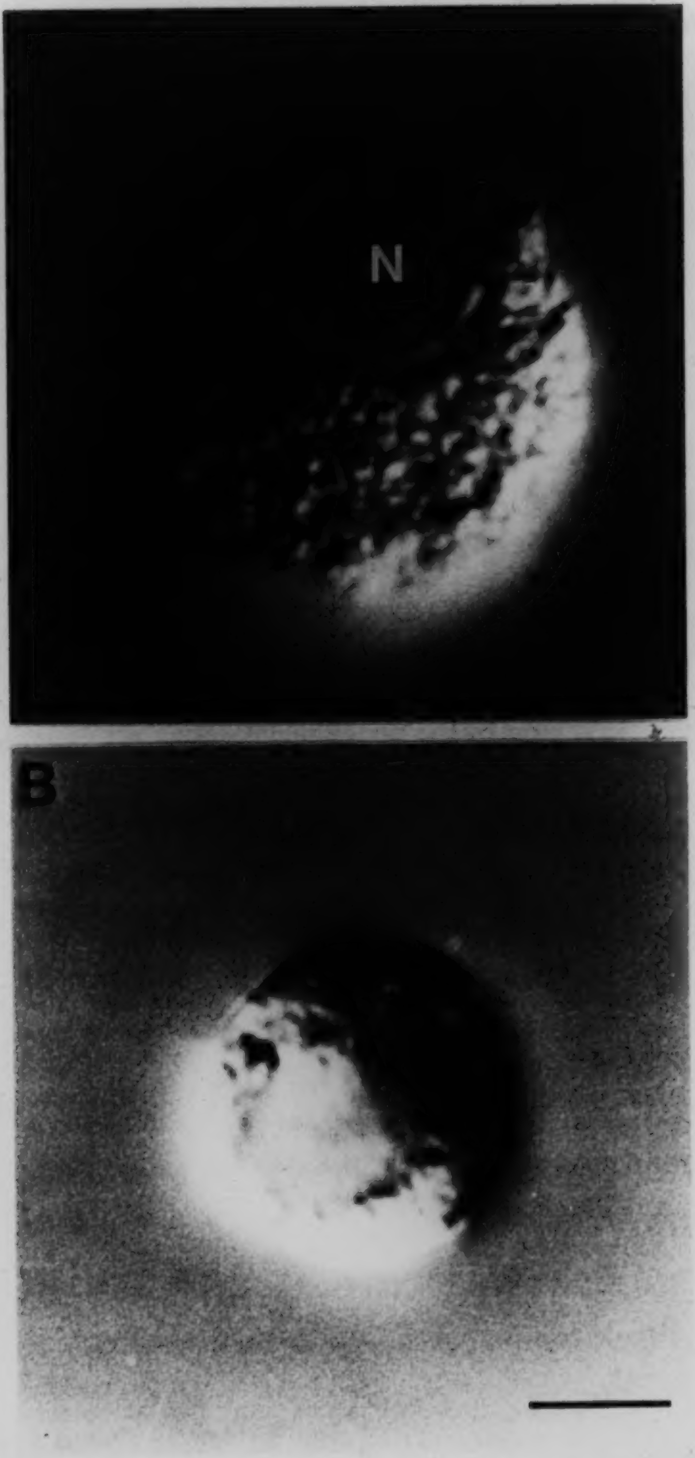


Fig. 2.6. Morphology of lactotrophs as revealed by DIC microscopy. The large irregularly shaped nucleus (*N*) is readily apparent in immunofluorescently labelled (A) and live (B) lactotrophs. x3200. *Bar*: 5 µm



Chapter 3

ELECTRICAL MEMBRANE PROPERTIES AND IONIC CURRENTS IN GOLDFISH GONADOTROPHS[†]

INTRODUCTION

Alterations in pituitary cell plasma membrane electrical activity play an important role in the control of pituitary hormone-release responses to various hypothalamic releasing or inhibitory factors (Stojilkovic and Catt, 1992; Tse and Hille, 1992, 1993; Vaudry *et al.*, 1994). In mammals, activation of voltage-dependent Ca^{2+} channels and the subsequent rise in $[Ca^{2+}]_i$ are important components in mediating pituitary hormone secretion, including the gonadotropin response to GnRH (Chang and Jobin, 1994a; Stojilkovic *et al.*, 1994). Furthermore, in rat gonadotrophs, GnRH-induced cytosolic Ca^{2+} oscillations initiate transient plasma membrane hyperpolarizations due to the periodic activation of apamin-sensitive, Ca^{2+}-activated K^+ channels. The transient membrane hyperpolarizations are usually followed by a few Na^+- and Ca^{2+}-dependent action potentials (Tse and Hille, 1993; Stojilkovic *et al.*, 1994). Other K^+ conductances have also been shown to be involved in the neuroendocrine regulation of rat lactotrophs (Lledo *et al.*, 1990), amphibian melanotrophs (Valentijn *et al.*, 1991; Vaudry *et al.*, 1994), and ovine somatotrophs (Chen *et al.*, 1994a); therefore, a variety of ionic channels are involved in the regulation of pituitary cell function.

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Pharmacological studies indicate that GnRH-induced Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channels and the resulting increase in $[Ca^{2+}]_i$ concentration mediates GTH-II secretion from the goldfish anterior pituitary (Chang *et al.*, 1993; Jobin and Chang, 1992a, 1992b). In addition, preliminary studies suggest that dopamine D_2 inhibition of GTH-II secretion involves an inhibition of Ca^{2+} influx (Chang *et al.*, 1993). Although voltage-dependent Ca^{2+} channels are critical components involved in the regulation of GTH-II secretion in goldfish, voltage-gated Ca^{2+} currents or any other ionic currents have not been characterized in teleostean gonadotrophs. Consequently, in the present chapter, whole-cell voltage- and current-clamp recording techniques were utilized to study the bioelectrical properties of identified goldfish gonadotrophs. The results presented here demonstrate that goldfish gonadotrophs possess single classes of voltage-dependent Na^+ and Ca^{2+} currents, multiple classes of K^+ currents, and electrical excitability. This study is the first to describe the ion channel properties in teleostean gonadotrophs. In addition, it provides the foundation for further investigation of the electrical membrane properties involved in GnRH and dopamine regulation of GTH-II secretion.

MATERIALS AND METHODS

Animals

Common goldfish (*Carassius auratus*; 8-13 cm in body length) were purchased from Grassyforks Fisheries (Martinsville, IN, USA) and held in flow-through aquaria (1800 l). Fish were maintained at 17-20 °C on a simulated natural photoperiod (adjusted weekly according to the times of sunset and sunrise in Edmonton, AB, Canada) and fed to satiation with commercial fish food daily. Fish were acclimated to the above conditions for at least seven days prior to use. Serum GTH-II concentrations and GnRH receptor content in the pituitary gland change throughout the seasonal reproductive cycle of the goldfish (Habibi *et al.*, 1989). Therefore, to facilitate future comparisons, the time of year in which the currents were recorded are reported in the figure legends. In general, goldfish in the temperate zone become mature (prespawning) by late winter or early spring. After spawning (April and May), the gonads undergo regression and remain regressed throughout the summer and early fall. Gonadal recrudescence occurs over the winter months .

Cell Culture and Identification

Male and female goldfish were anesthetized in 0.05% tricaine methanesulfonate prior to decapitation. Pituitaries were removed and placed in dispersion medium (Medium 199 with Hank's salts, 25 mM HEPES, 2.2 g/l $NaHCO_3$, 0.3% bovine serum albumin, 100 000 U/l penicillin, and 100 mg/l streptomycin, pH 7.2; Gibco, Grand Island, NY, USA). Pituitary cells were dispersed using the controlled trypsin/DNAse treatment procedure described by Chang *et al.*, (1990a). Dispersed pituitary cells were cultured for 16 to 48 hr in poly-L-lysine (0.01 mg/ml) coated glass-bottom Petri dishes containing culture medium (Medium 199 with Earle's salts, 1 % horse serum, 25 mM HEPES, 2.2 g/l $NaHCO_3$, 100 000 U/l penicillin and 100 mg/l streptomycin, pH 7.2; Gibco) under 5% CO_2, saturated humidity, and at 28 °C. These culture conditions have been used routinely to provide cells that are responsive to neuroendocrine regulators in hormone-release studies (Chang *et al.*, 1990a). Gonadotrophs were identified by their unique cellular morphologies when viewed under Nomarski DIC microscopy (Chapter 2).

Electrophysiological Recordings

Electrophysiological studies of identified goldfish gonadotrophs were performed at room temperature (18 - 20 °C) using whole-cell, gigaohm-seal (Hamill *et al.*, 1981) or nystatin-perforated patch (Korn and Horn, 1989) recording techniques. Current- and voltage-clamp recordings were carried out using a Dagan 3900 (Minneapolis, MN) integrating patch-clamp amplifier and were low-pass filtered at 2 KHz using a four-pole Bessel filter. Patch electrodes, fabricated from borosilicate glass (1.5 mm OD; World Precision Instruments, Sarasota, FL) using a Flaming Brown horizontal puller (P-87; Sutter Instruments, Novato, CA), were heat polished to a final tip resistance of 3 to 8 MΩ and then coated with dental wax to reduce pipette capacitance. Prior to seal formation, liquid junctional potentials were cancelled. Following the formation of a giga-seal (seal resistance = 6.8 ± 1.0 GΩ; range 1.3 - 20 GΩ, *n* = 56), pipette capacitance was neutralized and the patch membrane was ruptured using gentle suction. Series resistance (Rs) was calculated to be 26.1 ± 2.4 MΩ from the relation $Rs = \tau/C_m$, where τ is the decay time constant of the capacitive current transient following a 10 mV hyperpolarizing voltage pulse ($\tau = 0.14 \pm 0.06$ msec; mean ± SEM; *n* = 15) and C_m is the cell capacitance. The cell capacitance was determined by integrating the capacity current transient during a 10 mV hyperpolarizing voltage pulse (V_m) and applying the relation $C_m = q/V_m$. Cell capacitance was in the range of 4 - 7 pF (5.2 ± 0.4 pF; *n* = 15). Series resistance compensation was optimized and usually ranged between 10 and 18 MΩ. Current records were corrected for linear leakage and capacitance using a P/-4 procedure (Bezanilla and Armstrong, 1977). Pulse generation, and data acquisition were carried out using an AT 486 2DX compatible computer equipped with a digidata 1200 interface in conjunction with pCLAMP programs (Axon Instruments, Foster city, CA).

In some experiments, perforated-patch recordings were used to minimize current rundown and disruption of the cellular cytoplasm (Korn and Horn 1989). A nystatin stock solution (50 mg/ml) was prepared in dimethyl sulfoxide (DMSO) and stored for up to 1 week at -20 °C. Just prior to use, the stock solution was diluted in pipette solution and sonicated for 30 sec to yield a final nystatin concentration of 160 μg/ml. Patch electrodes used for perforated-patch recordings were fabricated as described above and had a final tip resistance of 3 to 5 MΩ. Pipette tips were briefly immersed in nystatin-free solution and then backfilled with the nystatin-containing solution. After obtaining a GΩ seal and neutralizing pipette capacitance, the pipette potential was set to -70 mV and a 10 mV hyperpolarizing voltage step was given to monitor access resistance (Fig. 3.1). An average series resistance of 29.6 ± 1.9 MΩ (*n* = 41) was reached 10 min. following the formation of a giga-seal and remained stable for up to one hour. Series resistance and membrane capacitance were calculated as described above using $\tau = 0.18 \pm 0.01$ msec and a cell capacitance of 6.4 ± 0.5 pF (range = 4 - 15 pF; *n* = 41).

Solutions

The composition (in mM) of the external solutions for the different recording conditions are listed as follows:

1. Total Current: modified Hank's balanced salt solution (HBSS), consisting of 140 NaCl, 5 $CaCl_2$, 5.4 KCl, 0.4 KH_2PO_4, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 0.3 $NaHPO_4$, 4.2 $NaHCO_3$, 5.5 glucose, 15 HEPES (pH adjusted to 7.2 with NaOH).

2. Na^+ currents: $CdCl_2$ (50 μM) with either Medium 199 (Hank's salts, 2.2 g/l $NaHCO_3$, 25 mM HEPES, 100 000 U/l penicillin, 100 mg/l streptomycin, 0.1% bovine serum albumin, pH adjusted to 7.2 with NaOH; Gibco) or HBSS.

3. K^+ currents: modified HBSS in which NaCl was replaced with equimolar choline chloride (pH adjusted to 7.2 with KOH).

4. Ba^{2+} currents (through voltage-sensitive Ca^{2+} channels): 120 choline chloride, 1.26 $CaCl_2$, 20 $BaCl_2$, 1.0 $MgCl_2$, 5.5 glucose, 10 HEPES (pH adjusted to 7.2 with tetraethylammonium [TEA]-OH).

The osmolarity of all external solutions used in this study differed by less than 10% from that measured in goldfish plasma (≈ 300 mOsm; range 280 to 320 mOsm; J.P. Chang and J.A. Wiggs, unpublished observations). The bath was continuously perifused at a rate of 2 ml/min using a gravity driven superfusion system and the outflow was placed near the cell, resulting in complete solution exchange around the cell within 10 sec.

The composition (in mM) of the electrode solutions for the different recording conditions are listed as follows:

1. Total current: 120 K-aspartate, 20 KCl, 2 $MgCl_2$, 10 HEPES and 150 - 200 µg/ml nystatin (pH adjusted to 7.2 with TrisCl).

2. Na^+ currents: 75 CsCl, 75 cesium-glutamate, 0.1 EGTA, 10 HEPES, 2.5 Mg-ATP and 0.4 Na-GTP (pH adjusted to 7.2 with CsOH).

3. K^+ currents: 120 K-aspartate, 20 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 10 HEPES, 2.5 Mg-ATP and 0.4 Na-GTP (pH adjusted to 7.2 with TrisCl). For recordings of Ca^{2+}-activated K^+ currents the total current electrode solution was used.

4. Ba^{2+} currents: 120 cesium-glutamate, 20 TEA-Cl, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 10 HEPES, 2.5 Mg-ATP and 0.2 Na-GTP (pH adjusted to 7.2 with TEA-OH). With 11 mM EGTA and 1 mM Ca^{2+} in the electrode solution, the Ca^{2+} concentration was estimated to be $< 2.0 \times 10^{-8}$ M (MaxChelator, v 6.5; C. Patton, Pacific Grove, CA).

Drugs

Tetrodotoxin (TTX), nifedipine and S(-)-Bay K 8644 (Research Biochemicals Incorporated, Natick, MA) were dissolved in ethanol. Apamin (Research Biochemicals Incorporated) was dissolved in 0.05 M acetic acid. Aliquots of stock solutions were stored at -20 °C until use, when they were diluted to final concentrations in saline; the highest concentrations of ethanol and acetic acid were 0.01% and 0.05 mM, respectively. TEA and 4-aminopyridine (4-AP; Sigma, St. Louis, MO) were prepared in saline immediately prior to use.

Data Analysis

Data analysis was performed using an AT 486 2DX compatible computer in conjunction with Clampfit (Axon Instruments, Foster city, CA). In some cases, the current-voltage relationships were fitted with the Boltzmann relation;

$$[1] \qquad I/I_{max}=I_{max}/\{1+\exp[(E-E_{1/2})/k]\}$$

where I_{max} = the maximal current, E = test potential, $E_{1/2}$ = the potential of 50% maximal current and k = slope factor. Exponential fits were performed using Clampfit. All values in the text are reported as mean ± SEM. Differences between groups were considered to be significant when $P < 0.05$ using Student's paired *t* test or ANOVA followed by Fisher's least significant difference test.

All voltages have been corrected for liquid junctional potentials ($E^o{}_L$) as described in Barry and Lynch (1991). Briefly, the $E^o{}_L$ for total current, isolated Na^+ current, isolated K^+ current and isolated Ba^{2+} current were measured according to Neher (1992) and were determined to be; 10.4 ± 0.6 mV, 8.8 ± 0.2 mV, 11.7 ± 0.7 mV and 12.3 ± 0.3 mV (*n*= 7), respectively. These values were similar to those estimated from the JPCalc program (Barry, 1994). Accordingly, all reported membrane potentials in the text and figures were corrected for E^{o}_{L} errors using the relation;

$$V_m = V_p - E^{o}_{L} \qquad [2]$$

where V_m = corrected membrane potential, V_p = pipette potential (Barry and Lynch, 1991). The zero current level in all voltage-clamp figures and the unstimulated membrane potential in all current-clamp figures are indicated by dotted lines.

RESULTS

General Electrophysiological Properties

The general electrophysiological characteristics of goldfish gonadotrophs were studied under perforated-patch whole-cell recording conditions. Gonadotrophs had a resting membrane potential of -61.3 ± 2.1 mV (range = -42 mV to -80 mV; *n* = 19). At their resting membrane potential, 70% of gonadotrophs exhibited small, non-overshooting action potentials in response to depolarizing current pulses from 1 to 5 pA (Fig. 3.2 A; lower panel). In these cells, action potentials were triggered at membrane potentials between -43 and -25 mV and had an amplitude of 44.9 ± 4.2 mV with a duration of 10.0 ± 2.1 msec (*n* = 5) at one-half amplitude. In all gonadotrophs with a membrane potential of -80 mV, one or more action potentials were evoked by injection of depolarizing current pulses from 1 to 5 pA (Fig. 3.2 A;

upper panel and Fig. 3.2 B). From this membrane potential, action potentials were triggered at potentials between -50 and -36 mV and had an amplitude of 80.5 ± 5.8 mV with a duration of 3.2 ± 0.4 msec (n = 7) at one-half amplitude. In gonadotrophs which exhibited multiple action potentials in response to a depolarizing current pulses, there was a subsequent decrease in action potential amplitude and an increase in their duration compared to the first action potential (Fig. 3.2 B). The induced action potentials were sensitive to TTX. In four cells tested, application of 1 μM TTX reduced the mean action potential amplitude from 53.6 ± 3.2 mV to 38.5 ± 1.7 mV (paired t-test, $P <$ 0.05) and increased the action potential duration at one-half amplitude from 4.3 ± 0.8 msec to 8.3 ± 0.6 msec ($P < 0.05$; Fig. 3.3 A). In these same cells, 1 μM TTX abolished the inward Na^+ current under voltage-clamp recording conditions (Fig. 3.3 B). In 43% of the gonadotrophs examined (6 out of 14), spontaneous action potential activity was observed (Fig. 3.2 C). The remaining gonadotrophs exhibited spontaneous voltage fluctuations which did not give rise to action potentials. The membrane potential change in response to hyperpolarizing current injections from -5 to 0 pA was linear and the slope of the voltage-current relation indicated a mean input resistance of 8.2 ± 1.6 GΩ (range 4 - 14.5 GΩ; n = 7: Figure 3.2 D). Under voltage-clamp recording conditions, depolarizing voltage steps from a holding potential of -80 mV elicited a fast-activating inward current followed by an outward current (Fig. 3.2 E). To characterize the individual inward and outward currents, they were isolated using ionic substitutions and pharmacological techniques.

Characterization of Outward Currents

Using standard whole-cell recording techniques, voltage-sensitive K^+ currents in goldfish gonadotrophs were isolated by replacing extracellular Na^+ with equimolar choline chloride. In addition, to eliminate any possible Ca^{2+}-activated K^+ currents, extracellular Ca^{2+} entry was blocked by the addition of 0.05 mM $CdCl_2$. The ionic selectivity of the outward current was established from the reversal potential of tail currents. In the presence of 5.8 mM external K^+ and 140 mM K^+ in the pipette, the current reversed at -84.3 ± 0.2 mV (Fig. 3.4; n = 3). This value is close to the K^+ equilibrium potential calculated from the Nernst equation (-80.2 mV; 18 °C) at an intracellular K^+ concentration of 140 mM. The ionic selectivity of the current was further demonstrated by the abolition of the outward currents when K^+ in the pipette was replaced by the K^+ current blocker Cs^+.

To examine the voltage-dependent outward K^+ current, cells were given a conditioning pre-pulse to -132 mV for 500 msec prior to giving a series of 1-sec depolarizing voltage steps from -82 mV to +78 mV (Fig. 3.5 A). The threshold of activation for the total outward K^+ current was -54.9 ± 3.3 mV (n = 7), which is slightly more depolarized than the resting membrane potential of these cells. Voltage steps to potentials between -52 and +8 mV elicited a relatively slowly activating (time to peak > 20 msec) and inactivating current. Voltage steps to potentials more positive than -20 mV elicited a fast-activating transient component followed by a sustained lower amplitude K^+ current. At command potentials of +38 mV, peak outward currents reached an amplitude of 495.4 ± 56.1 pA (range: 332 to 767 pA; n = 7) within 12.0 ± 1.5 msec (range; 7 to 19 msec; n = 7). At the same command potential, the second component of the outward current inactivated slowly during a 1-sec depolarizing pulse and reached an average amplitude of 245.4 ± 16.5 pA (range: 194 to 306 pA; n = 7).

To determine the voltage-dependence of activation and inactivation of the K^+ current, a two-pulse protocol was used (Bezanilla and Armstrong, 1977). Steady-state inactivation was determined by measuring the remaining current elicited by a test pulse to +38 mV following a series of 1-sec prepulse potentials from -132 to +8 mV (Fig. 3.5 B). The voltage-dependence of activation was determined by the maximal outward current elicited during the 1-sec pre-pulse potentials from -132 to +8 mV. The resulting currents were normalized to the maximal outward current and plotted against pre-pulse potentials. The threshold for activation of the K^+ current was around -52 mV. Following a 1-sec depolarizing pre-pulse to membrane potentials more depolarized than -32 mV, only half of the maximal current could be ellicited by a subsequent command potential to +38 mV (Fig. 3.5B). The inactivation curve could not be fitted with a single Boltzmann relation. The activation and inactivation kinetics of the K^+ current indicate that multiple voltage-dependent K^+ current subtypes are present in identified goldfish gonadotrophs. To characterize the various voltage-dependent K^+ currents present, their activation and inactivation kinetics, as well as their sensitivity to membrane potential, 4-AP and TEA were examined.

Transient potassium current. Cells were given a conditioning prepulse to -52 mV for 500 msec (holding potential = -82 mV) in order to inactivate the transient outward K^+ current (Rudy, 1988). Under these conditions, the peak K^+ current elicited by a command potential to +38 mV was reduced by 29.2 ± 0.5% ($P < 0.05$, paired t-test, $n = 4$) compared to that following a conditioning prepulse to -132 mV in the same cell (Fig. 3.6). In contrast, the late component of the outward K^+ current was not significantly reduced. The remaining K^+ current activated at command potentials more positive than -52 mV and inactivated slowly during the 1-sec depolarizing steps.

Subtraction of the K^+ current elicited during the various voltage steps in cells given a conditioning prepulse to -52 mV from cells conditioned at -132 mV revealed a fast-activating and inactivating K^+ current. To further characterize this transient K^+ current, I tested the effects of 4-AP, which has been demonstrated to block these transient currents in various mammalian pituitary cell types (Lledo *et al.*, 1990; Bosma and Hille, 1992) and in teleost cell preparations (Glassmeier *et al.*, 1992; Price *et al.*, 1993). Application of 2 mM 4-AP reversibly blocked the transient component of the K^+ current elicited at a command potential of +38 mV by 48.0 ± 4.3% ($n = 6$; $P < 0.05$, paired t-test; Fig. 3.7 A). In contrast, the sustained component was not significantly affected by 4-AP. Subtracting the current elicited in the presence of 4-AP from the total K^+ current revealed a fast-activating and inactivating current (Fig. 3.7 C). A voltage activation curve for this transient current (Fig. 3.7 D) was determined by measuring the peak current (I) activated by a given voltage (E). From these values the conductance (g) was calculated, using the relationship

$$g = I/(E-E_K). \tag{3}$$

For E_K, I used the K^+ equilibrium potential (-80.2 mV; 18 °C) calculated from the Nernst equation, assuming that the intracellular K^+ concentration was the same as that in the pipette solution (140 mM). The transient current activated at command potentials more positive than -62 mV, with full activation around +38 mV. The resulting curve could be fitted with a single Boltzmann relation, where $E_{1/2} = -13.2$ mV and $k = -22$. Therefore, half of the transient potassium current was activated at a membrane potential of -13.2 mV. The combination of the voltage-sensitivity, fast activation and inactivation kinetics, and the specific blockade of the transient K^+ current by

4-AP, clearly demonstrates that identified goldfish gonadotrophs contain currents similar to the I_A K^+ current first identified in molluscan neurons (Connor and Stevens, 1971).

Sustained potassium current. Elimination of the I_A-type current by depolarizing prepulses to -52 mV or the addition of 2 mM 4-AP uncovered a slowly inactivating current elicited by command potentials more positive than -52 mV (Fig. 3.6 and 3.7). This sustained outward current was insensitive to 2 mM 4-AP but was reduced by 49.0 ± 2.8% ($P < 0.05$; $n = 4$) at a command potential of +38 mV by externally applied 5 mM TEA (Fig. 3.8). Likewise, externally applied 5 mM TEA reduced the transient outward current by 53.0 ± 0.3% ($P < 0.05$; $n = 4$). The effect of 5 mM TEA on both the transient and sustained current was reversible. On the basis that the sustained current is relatively resistant to inactivation during the 1-sec depolarizing pulses and its insensitivity to depolarizing pre-pulses, as well as 4-AP, we have determined that at least part of the sustained current is similar to the delayed rectifier (I_K) current characterized in other cell types (Rudy, 1988). In addition, as in other cell types (Rudy, 1988), the delayed rectifier current in goldfish gonadotrophs is sensitive to externally applied TEA.

Calcium-activated potassium currents. Ca^{2+}-activated K^+ currents have been demonstrated to play an important role in mediating GnRH-stimulated current oscillations in rat (Kukuljan *et al.*, 1992; Tse and Hille 1992, 1993) and ovine pars distalis gonadotrophs (Heyward *et al.*, 1993). These currents have been shown to be sensitive to the bee venom apamin (Kukuljan *et al.*, 1992; Tse and Hille 1992), which blocks the small conductance type of Ca^{2+}-activated K^+ currents (Castle *et al.*, 1989). In order to determine the presence of Ca^{2+}-activated K^+ channels, 5 mM Ca^{2+} was added to the bath and Na^+ was replaced by choline. In the presence of externally applied 1 μM apamin, a slight reduction of peak outward current was observed at command potentials from +8 to +72 mV and were maximal at a command potential of +38 mV (Fig. 3.9 A). However, no significant differences occurred when test currents were compared to control (pre-apamin) currents or to currents observed after washout of apamin (maximal reduction of 8.6 ± 3.5%; *n* = 5; $P >$ 0.05 vs. zero change). Similarly, no consistent effects of 1 μM apamin on induced action potential activity were observed (*n* = 4; data not shown). Furthermore, when Ca^{2+} influx was blocked by the addition of 50 μM $CdCl_2$, neither the amplitude nor current-voltage relationship of the outward current recorded under perforated-patch recording conditions were altered (*n* = 4; Fig. 3.9 B). Under the conditions outlined here, we could not clearly demonstrate the presence of a Ca^{2+}-activated, apamin-sensitive K^+ channels in identified goldfish gonadotrophs.

Characterization of Sodium Currents

For the characterization of voltage-dependent Na^+ currents, outward currents were blocked by substituting K^+ with Cs^+ in the electrode solution and inward Ca^{2+} currents were blocked by the addition of 50 μM $CdCl_2$ to the bath solution. Inward Na^+ currents were activated at command potentials more positive than -49 mV and reached a maximum amplitude near -9 mV (Fig. 3.10 A). Under these recording conditions, the maximal inward Na^+ current elicited by a 100 msec depolarizing voltage step to -9 mV from a holding potential of -79 mV averaged 347 ± 39 pA (range: 112 – 505 pA; *n* =9). The currents elicited by voltage steps displayed both fast activation and inactivation kinetics, typical of voltage-dependent Na^+ currents. In the example shown (Fig. 3.10), the current activated rapidly between command potentials of -29 and +11 mV (time to peak was 1.6 msec to 0.5 msec). The inactivation time constant was determined by fitting a single exponential curve to the falling phase of the Na^+ current. In the example shown, the current inactivated rapidly, having a time constant of decay from 2.7 msec at -29 mV to 0.5 msec at +11 mV (Fig. 3.10 A). The maximal Na^+ current was reduced by 97.2 ± 1.0% by 250 nM TTX ($P < 0.05$, ANOVA followed by Fishers' LSD test, $n = 4$) and recovered to 55.6 ± 1.1% of control levels (Fig. 3.11).

As indicated earlier, larger action potentials were observed in gonadotrophs held at -80 mV rather than at the resting membrane potential prior to the application of 1 to 5 pA depolarizing current pulses. These results suggest that, at rest, a proportion of the Na^+ channels are not available for activation. To determine the proportion of the total Na^+ current available for activation at the resting membrane potentials of identified gonadotrophs, I examined the voltage-dependence of steady-state inactivation of the Na^+ current using a two-pulse protocol (Bezanilla and Armstrong, 1977). Cells held at -79 mV were subjected to pre-pulse potentials ranging from -99 to -9 mV for 100 msec after which a test pulse to -9 mV was given (Fig. 3.10 B). The normalized test current from 6 diffrent cells was plotted against prepulse potentials and the resulting curve was fit with a Boltzmann relation (equation 1; $E_{1/2} = -50.8$ mV and $k = 8.8$). The estimated membrane potential for half-maximal steady-state inactivation (-50.8 mV) is slightly more depolarized than the resting membrane potential of these cells, indicating that greater than 50% of the Na^+ channels are available for activation at rest.

Characterization of Barium Currents

Results from pharmacological studies have implicated the involvement of dihydropyridine-sensitive Ca^{2+} channels in the mediation of GnRH-stimulated GTH-II secretion from dispersed goldfish pituitary cells (Chang *et* *al.*, 1993, 1996). Therefore, the voltage-dependent Ca^{2+} currents in goldfish gonadotrophs were characterized using whole-cell voltage-clamp recording techniques. In the present study, Ba^{2+} was used as the charge carrier instead of Ca^{2+} for four reasons: 1. to minimize current rundown commonly associated with Ca^{2+} currents; 2. Ba^{2+} normally permeates more readily than Ca^{2+} through Ca^{2+} channels; 3. Ba^{2+} is relatively impermeant through Na^+ channels; and 4. Ba^{2+} blocks certain types of outward K^+ currents (Bean, 1992). However, to ensure that the behavior of Ba^{2+} as the charge carrier through voltage-sensitive Ca^{2+} channels is similar to that of Ca^{2+}, comparisons between currents carried by either Ba^{2+} or Ca^{2+} were made.

In identified goldfish gonadotrophs, a larger current amplitude was observed in the presence of 10 mM external Ba^{2+} than in the presence of 10 mM external Ca^{2+} (Figure 3.12 A). For example, during a 40 msec voltage step to -12 mV (holding potential = -92 mV), the current amplitude in the presence of 10 mM Ca^{2+} and Ba^{2+} was -32.1 ± 6.1 pA and -73.4 ± 8.1 pA, respectively ($n = 5$). Although a larger current amplitude was observed in the presence of external Ba^{2+}, the activation and inactivation properties of the currents carried by either Ba^{2+} or Ca^{2+} were similar (Fig. 3.12 B; $n = 5$). In particular, the time course of inactivation for both the Ca^{2+} and Ba^{2+} current elicited by a 200 msec voltage-step to -12 mV (holding potential = -92 mV) could be fitted with a single exponential with similar time constants of 106.6 ± 13.1 msec and 95.2 ± 16.9 msec, respectively ($P > 0.05$; $n = 5$). As the activation and inactivation properties of the Ba^{2+} and Ca^{2+} currents were similar, Ba^{2+} was used as the charge carrier instead of Ca^{2+} for the remainder of the study. To ensure that the currents carried by Ba^{2+} were dependent on the external Ba^{2+} concentration, the maximal Ba^{2+} current amplitude in either 10 mM or 20 mM Ba^{2+} were compared (Fig. 3.12 A). During a 40 msec voltage step to -12 mV (holding potential = -92 mV) the current amplitude in the presence of 10 and 20 mM Ba^{2+} were -73.4 ± 8.1 pA and -99.6 ± 12.5 pA, respectively ($n = 5$).

In the presence of 20 mM external Ba^{2+}, command potentials more depolarized than -52 mV elicited an inward Ba^{2+} current which was resistant to inactivation during the 200 msec command pulse (Fig. 3.13 A). Under these recording conditions, maximal inward Ba^{2+} currents elicited by a depolarizing voltage step to -12 mV from a holding potential of -92 mV were -129.9 ± 12.1 pA (range: -64 to -172 pA; $n = 9$) for peak (0 to 20 msec) and -129.4 ± 12.1 pA (range: 81 to 181 pA; $n = 9$) for sustained (190 to 200 msec) currents. The activation time course could be fitted with a single exponential curve having a time constant of 2.9 ± 0.3 msec at -32 mV, 2.6 ± 0.4 msec at -22 mV and 1.5 ± 0.04 msec at -12 mV. To determine the proportion of the Ca^{2+} channels available for activation at the resting membrane potential of these cells, I examined the steady-state inactivation properties of the Ba^{2+} current using a two-pulse protocol. The normalized currents from 6 diffrent cells elicited during test pulses to -12 mV were plotted against prepulse potentials and were fitted with a Boltzmann relation (equation 1), where $E_{1/2} = -16.7$ mV and $k = 20$ (Fig. 3.13 B). The membrane potential at which half maximal inactivation occurs is more depolarized than the resting membrane potential (≈ -60 mV) of these cells. Therefore, at rest, a large proportion (> 80%) of the Ca^{2+} channels are available for activation. The relatively high activation range and inactivation resistant properties of the Ba^{2+} current suggest that the Ca^{2+} channels present in these cells resemble the L-type channels found in other cell types (Tsien *et al.*, 1988). In addition, the similarity in the current-voltage relationship of the peak and sustained current indicate that T-type voltage-sensitive Ca^{2+} channels are not present in these cells (Fig. 3.13 A).

To further investigate the properties of the voltage-dependent Ba^{2+} currents, the effects of $CdCl_2$, and the 1,4-dihydropyridine Ca^{2+} channel activator, SEC(-)-Bay K 8644, and antagonist, nifedipine, were investigated. In the presence of 50 µM $CdCl_2$, the current elicited by a 40 msec depolarizing voltage step from -92 mV to -12 mV was reduced by 83.8 ± 1.9% ($P < 0.05$; $n = 3$) and returned to control levels following washout (Fig. 3.14 A). Application of 10 µM nifedipine reversibly reduced the inward Ba^{2+} current elicited by a 40 msec voltage step to -12 mV from a holding potential of -92 mV by 39.2 ± 12.7% (Fig. 3.14 B; $n = 3$). In contrast, in the presence of 1 µM S(-)-Bay K 8644, the inward Ba^{2+} current was increased by 15.8 ± 2.4% ($P < 0.05$; $n = 4$) and returned to control levels following washout (Fig. 3.14 C). This increase in Ba^{2+} current only occurred between voltage steps from -52 to -2 mV; current resposnes to voltage steps more depolarized than -2 mV were not significantly affected by Bay K 8644. The sensitivity of these currents to 1,4-dihydropyridine Ca^{2+} channel agonists and antagonists confirm that they resemble the L-type Ca^{2+} channels found in other cell types (Tsien *et al.*, 1988).

DISCUSSION

Electrical Membrane Properties of Identified Goldfish Gonadotrophs

In this chapter, the electrical membrane properties and ionic currents in identified goldfish gonadotrophs were characterized using whole-cell current- and voltage-clamp recordings. Identified goldfish gonadotrophs in primary cell cultures of mixed populations of dispersed goldfish pituitary cells have a resting membrane potential near -60 mV. This value is slightly more hyperpolarized than those reported for gonadotrophs from both male (-46 mV; Tse and Hille, 1993) and ovariectomized (around -50 mV; Stojilkovic *et* *al.*, 1992) female rats. In addition, the resting membrane potential reported here is more hyperpolarized than those values reported for general populations of goldfish pituitary cells (-35 mV; Price *et al.*, 1993) and for identified gonadotrophs under standard whole-cell recording conditions (-48.4 ± 1.7 mV; *n* = 14; F. Van Goor, unpublished observations). This may be due in part to the present use of perforated-patch recordings rather than standard whole-cell recordings (Price *et al.*, 1993). Perforated-patch recordings prevent the disruption of cytoplasmic conditions and second messenger activities which may contribute to the resting membrane potential. For example, Na^+-Ca^{2+} exchange has been demonstrated to be affected by standard whole-cell recording conditions (Korn and Horn, 1989). Further discrepancies in the resting membrane potentials reported in this study from those in other studies, such as Price *et al* (1993), may result from uncompensated liquid junction potentials (Barry, 1994). It is important to note that resting membrane potential may be different from cells *in vivo*, as it may be altered by extrinsic factors not accounted for in culture. Nevertheless, the resting membrane potential reported here is similar to those reported for gonadotrophs in other species recorded under similar conditions.

Similar to gonadotrophs from ovariectomized female rats (Stojilkovic *et al.*, 1992), rat somatotrophs (Chen *et al.*, 1990) and melanotrophs (Stack and Surprenant, 1991), as well as frog melanotrophs (Valentijn *et al.*, 1991), but unlike bovine somatotrophs (Mason and Rawlings, 1988) and ovine gonadotrophs (Mason and Waring, 1985), goldfish gonadotrophs exhibited spontaneous action potential firing. In addition, in response to depolarizing current injections, one or more action potentials could be elicited in goldfish gonadotrophs. In these cells, TTX reduced the amplitude of the action potentials and increased their duration, indicating that the action potentials are partially dependent on Na^+ influx through voltage-dependent, TTX-sensitive Na^+ channels. However, action potential activity persists even after Na^+ currents have been eliminated by TTX, suggesting that Ca^{2+} currents also contribute to action potential activity in identified goldfish gonadotrophs.

Two other observations are consistent with the involvement of both Na^+ and Ca^{2+} channels during action potential activity in these cells. Firstly, although action potentials could be elicited in most gonadotrophs held at rest, they were lower in amplitude than those elicited from more hyperpolarized membrane potentials. This is likely due to the voltage-dependent properties of the Na^+ channels present in the goldfish gonadotrophs, as about half of the Na^+ channels are inactivated at rest (see discussion of Na^+ channels below). Secondly, greater than 80% of the Ca^{2+} channels are available for activation at the resting membrane potential of identified goldfish gonadotrophs. Moreover, in preliminary studies, the inorganic Ca^{2+} channel blocker $CdCl_2$ attenuated action potential activity in these cells (Van Goor, unpublished observations). These results strongly implicate the involvement of both Na^+ and Ca^{2+} influx during action potential activity in goldfish gonadotrophs.

Ionic Currents in Identified Goldfish Gonadotrophs

Goldfish gonadotrophs contain TTX-sensitive Na^+ currents. Similarly, voltage-dependent Na^+ currents were observed in general populations of goldfish pituitary cells (Price *et al.*, 1993), rat somatotrophs (Chen *et al.*, 1990) and a cell line of gonadotroph lineage (Bosma and Hille, 1992), as well as rat (Tse and Hille, 1993) and ovine (Mason and Rawlings, 1988) gonadotrophs. Compared to other vertebrate gonadotrophs, Na^+ currents in goldfish gonadotrophs activated at similar potentials to that of male rats (Tse and Hille, 1993) but at more depolarized potentials than ovine gonadotrophs

(Mason and Sikdar, 1988) or gonadotroph cell lines (Bosma and Hille, 1992). The mid-point of voltage-dependent inactivation in goldfish gonadotrophs (-50.8 mV) is similar to the value reported for general populations of goldfish pituitary cells (-41 mV; Price *et al.*, 1993). This value is slightly more depolarized than the resting membrane potential of these cells, suggesting that, at rest, more than half of the Na^+ channels are available for activation. Compared to other vertebrate gonadotrophs, more Na^+ channels are available for activation at rest in cultured goldfish gonadotrophs. The mid-point of inactivation for goldfish gonadotrophs occurs at relatively more depolarized potentials than that of male rat gonadotrophs (-64 mV; Tse and Hille, 1993), ovine gonadotrophs (-70 mV; Mason and Rawlings, 1988) and the gonadotroph cell line (-92 mV; Bosma and Hille, 1992). Therefore, at normal resting membrane potentials, up to 93% of the Na^+ channels may be inactivated in mammalian gonadotrophs (Tse and Hille, 1993) as compared to less than 50% in the goldfish. This may reflect fundamental differences in plasma membrane events underlying GnRH action between these groups.

Similar to other pituitary cell types (Stojilkovic *et al.*, 1992), voltage-dependent Ca^{2+} currents were observed in identified goldfish gonadotrophs. In the present study, the voltage-sensitive Ca^{2+} currents were characterized by their kinetics, voltage-dependence, and pharmacological properties. To facilitate the study of voltage-dependent Ca^{2+} channels in goldfish gonadotrophs, Ba^{2+} was used as the charge carrier instead of Ca^{2+}. Calcium and Ba^{2+} currents through voltage-dependent Ca^{2+} channels have similar activation and inactivation characteristics, although larger current amplitudes were observed in the presence of external Ba^{2+}. Therefore, it is reasonable to extrapolate the findings shown here using Ba^{2+} as the charge carrier through voltage-dependent Ca^{2+} channels to the characteristics of

Ca^{2+}-flow through these channels. The Ba^{2+} currents in identified goldfish gonadotrophs activated rapidly at command potentials more positive than -40 mV, were resistant to inactivation during a 200 msec voltage-step, and were partially sensitive to the 1,4-dihydropyridine class of Ca^{2+} channel activator (Bay K 8644) and antagonist (nifedipine). In goldfish gonadotrophs, the ≈ 39% reduction in the maximal Ba^{2+} current amplitude induced by nifedipine was similar to that of rat melanotrophs (≈ 31% reduction, Stack and Surprenant, 1991) and male rat gonadotrophs (≈ 41% reduction, Tse and Hille, 1993). However, the partial dihydropyridine-sensitivity of the Ca^{2+} channels in goldfish gonadotrophs may indicate that other voltage-dependent Ca^{2+} channel subtypes are present. Whereas many of the properties of the voltage-dependent Ca^{2+} channels seen here are similar to those in other gonadotrophs (Mason and Sikdar, 1989; Bosma and Hille, 1992; Tse and Hille 1993), important differences were revealed. For example, the steady-state inactivation properties of the Ba^{2+} currents indicate that greater than 80% of the Ca^{2+} channels are available for activation at rest. In contrast, only 50% of the Ca^{2+} channels are available for activation at rest in rat gonadotrophs (Tse and Hille 1993). In addition, unlike ovariectomized rat gonadotrophs (Stojilkovic *et al.*, 1994), gonadotroph cell-lines (Bosma and Hille, 1992), and ovine pars tuberalis gonadotrophs (Mason and Waring, 1985), low-voltage activated transient inward currents were not observed in goldfish gonadotrophs when either Ca^{2+} or Ba^{2+} was used as the charge carrier through voltage-dependent Ca^{2+} channels.

In the present study, at least two voltage-dependent K^+ current subtypes were identified in goldfish gonadotrophs, including I_A-currents and delayed rectifier currents. The I_A-type currents found in these cells had similar kinetics and sensitivity to 4-AP and TEA as those reported in neurons (Rudy, 1988), other anterior pituitary cells (Lledo *et al.*, 1990; Bosma and Hille, 1992; Chen *et al.*, 1994a) and trout oligodendrocytes (Glassmeier *et al.*, 1992). Delayed rectifier currents similar to those reported here have also been identified in other pituitary cell types (Lledo *et al.*, 1990; Chen *et al.*, 1994a), as well as in general populations of dispersed goldfish pituitary cells (Price *et al.*, 1993). In contrast, unlike gonadotrophs from male (Tse and Hille 1992, 1993) and ovariectomized female rats (Kukuljan *et al.*, 1992), apamin-sensitive K^+ currents were not observed under our current recording conditions. In rat gonadotrophs, apamin-sensitive K^+ channels mediate GnRH-induced oscillations in membrane potential (Kukuljan *et al.*, 1992, 1994; Tse and Hille 1992, 1993) and are important in bringing voltage-sensitive Na^+ and Ca^{2+} channels out of steady-state inactivation (Tse and Hille, 1993). As mentioned above, in goldfish, a large proportion of voltage-sensitive Na^+ and Ca^{2+} channels are available for activation at rest and therefore transient hyperpolarizations due to the activation of apamin-sensitive K^+ channels may not be required to bring them out of steady-state inactivation.

In conclusion, goldfish gonadotrophs are electrically excitable and exhibit spontaneous action potential activity in some cases. Several different ionic currents were identified, including voltage-dependent Ca^{2+} and Na^+ currents, as well as multiple K^+ current subtypes. Apamin-sensitive K^+ currents, which mediate GnRH-induced oscillations of membrane potential in rat gonadotrophs, were not observed in goldfish gonadotrophs. This study is the first to confirm the presence of dihydropyridine-sensitive Ca^{2+} channels in goldfish gonadotrophs as first implicated by hormone-release studies. The role that each of these ionic currents plays in mediating the electrical membrane events underlying the neuroendocrine regulation of GTH-II release in goldfish gonadotrophs remains to be elucidated.

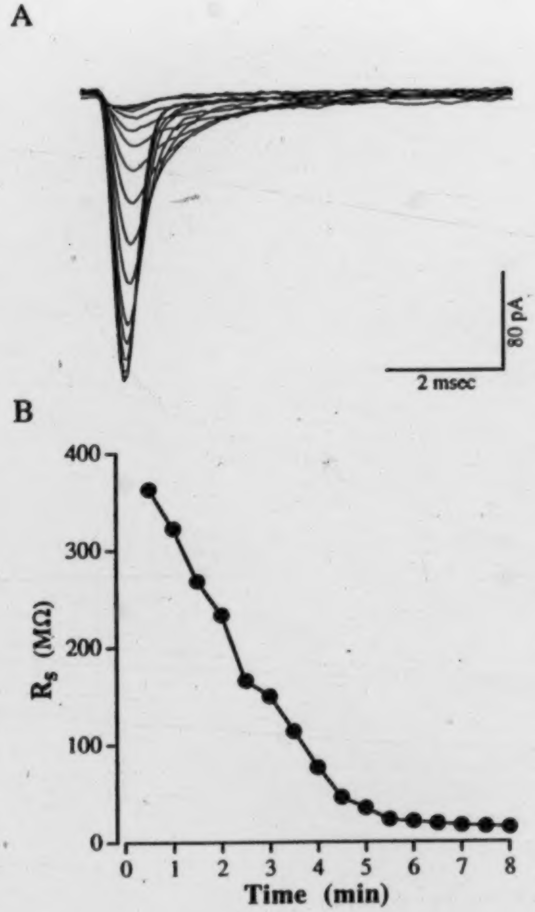

Fig. 3.1. Formation of electrical access to the cell interior using nystatin perforated-patch recordings in a representative gonadotroph. A. Superimposed membrane current responses to a 10-msec, 10 mV hyperpolarizing voltage pulse (holding potential = -70 mV), given every 30 sec immediately following the formation of a GΩ seal (time = 0). Pipette capacitance was neutralized prior to monitoring R_s. B. Time course of decrease in R_s following the formation of a GΩ seal. In this example, the steady-state R_s was calculated to be 15.7 MΩ using the relation $R_s = \tau/C_m$, where $C_m = 6.9$ pF and $\tau = 0.108$ msec.

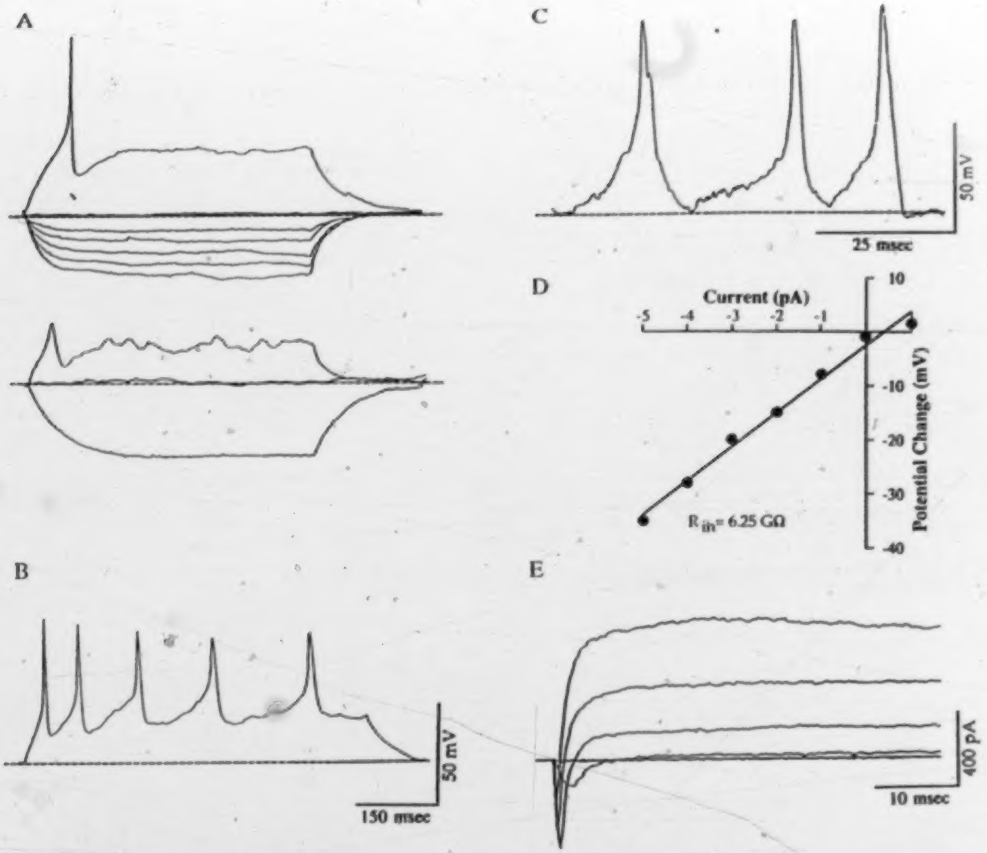

Fig. 3.2. Perforated-patch whole-cell current- and voltage-clamp recordings from an identified goldfish gonadotroph. A. Upper trace; current-clamp recording showing the membrane potential change in response to current injections between -5 and 0 pA, as well as a depolarizing current injection to +4 pA (membrane potential = -80 mV). Lower trace; current-clamp recording from the same cell as in A, showing the membrane potential change in response to current injections of -5, 0 and +4 pA (membrane potential = -50 mV). B. A current-clamp recording showing the membrane potential change in response to a depolarizing current injection to +4 pA (membrane potential = -80 mV) from a gonadotroph which exhibited multiple action potentials. C. Spontaneous action potential activity in a gonadotroph with a resting membrane potential of -45 mV. D. The voltage response to current injections between -5 and +1 pA (membrane potential = -80 mV) in the same cell as shown in A. The slope of the line gave an input resistance of 6.3 GΩ. E. Whole-cell voltage-clamp recordings of current responses under total current recording conditions (holding potential = -80 mV; voltage steps to -80, -30, -10, +10 and +40 mV are shown). All recordings were carried out from October to December.

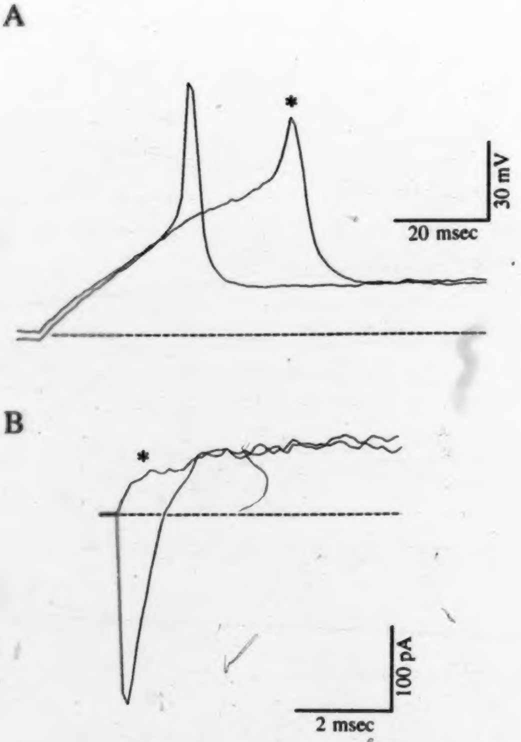

Fig. 3.3. TTX-sensitivity of induced action potential activity in an identified goldfish gonadotroph. A. Representative current-clamp recording illustrating the effects of 1 μM TTX (asterisk) on action potential amplitude and duration. Action potentials were induced by a depolarizing current injection to +1 pA from a membrane potential of -80 mV. B. Whole-cell voltage-clamp recordings of the effects of 1 μM TTX (asterisk) on the same cell as in A under total current recording conditions. Currents were elicited by a 40-msec voltage-step to 0 mV from a holding potential of -80 mV. All recordings were carried out from October to December.

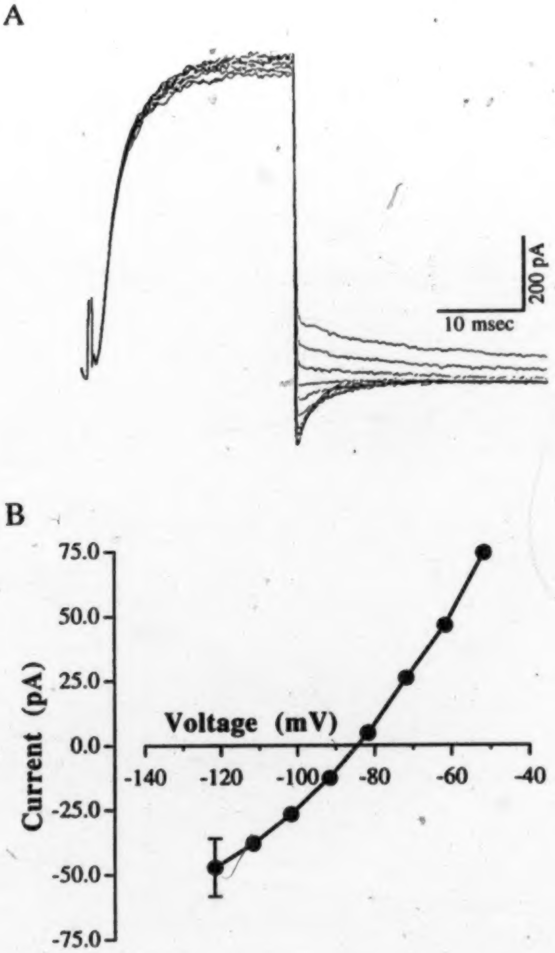

Fig. 3.4. Reversal potential of voltage-dependent outward currents in identified goldfish gonadotrophs. A. Superimposed current traces elicited by a 20-msec pre-pulse to +38 mV, followed by a series of 40-msec voltage steps from -132 mV to -42 mV in 10 mV increments. B. Current-voltage relationship of the peak tail current amplitude (mean ± SEM, = 3). The intersection of the curve with the x-axis indicates a reversal potential of -84 mV.

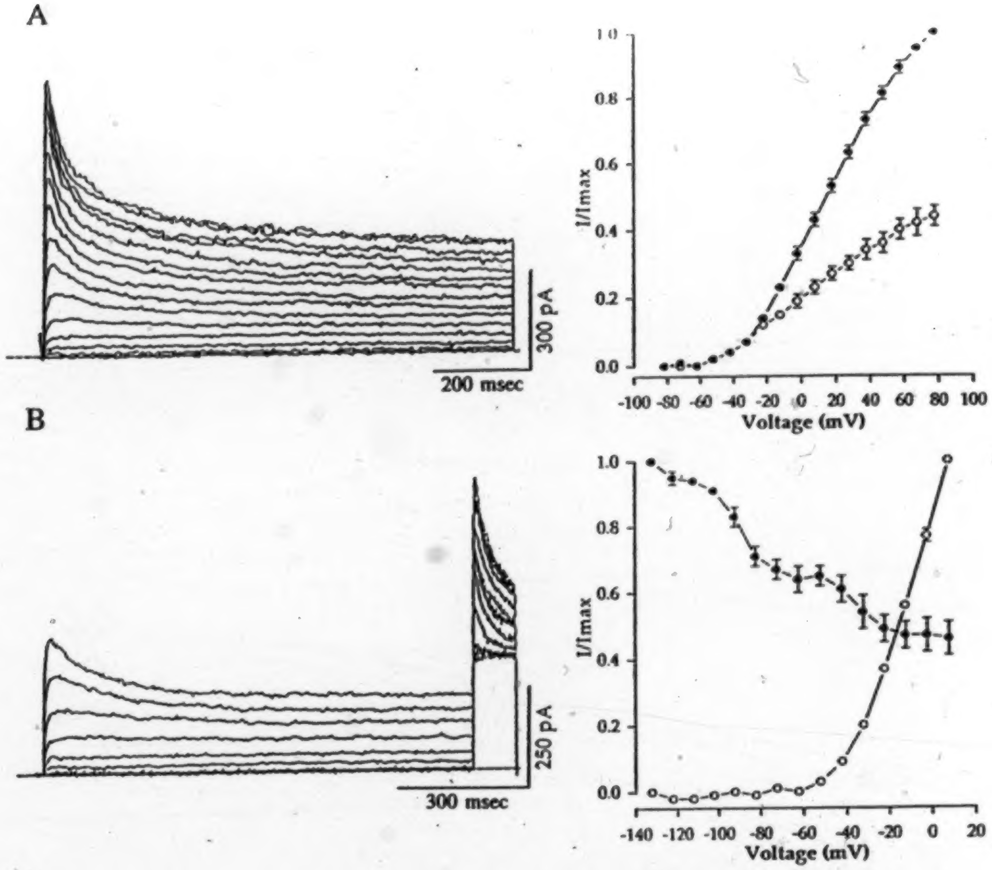

Fig. 3.5. Total outward voltage-dependent K+ current in identified goldfish gonadotrophs. A. Left panel: example currents elicited by 1-sec depolarizing voltage steps from -82 mV to +78 mV following a 500 msec conditioning pre-pulse to -132 mV. Right panel: current-voltage relation of the peak (0 - 20 msec; *filled circles*) and sustained (980 - 1000 msec; *open circles*) K+ current (mean ± SEM, *n* = 7). B. Steady-state activation and inactivation of total outward K+ current. For the determination of steady-state inactivation, 1-sec-long conditioning pre-pulses from -132 to +8 mV were given prior to a 20 msec command potential to +38 mV. Representative current traces are shown in the left panel. Right panel: the remaining peak current elicited during the command potential was plotted against the conditioning pre-pulse membrane potential (mean ± SEM, *n* = 10; *filled circles*). Activation curves were generated from the same cells (*open circles*). All currents in A and B were normalized to the maximal outward current. All recordings were carried out from September to January.

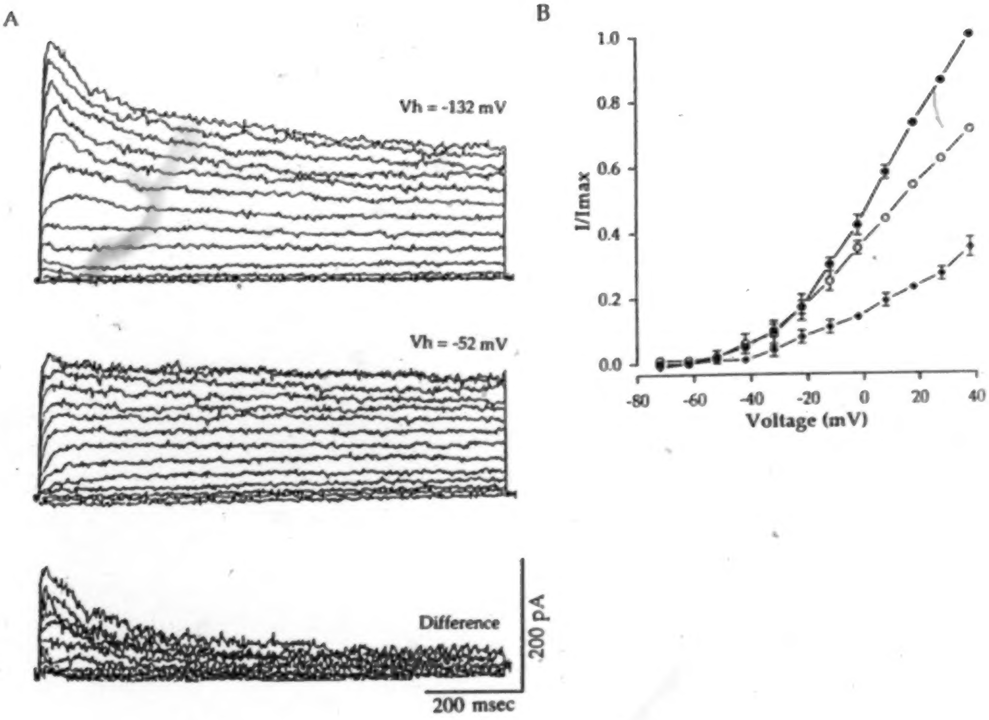

Fig. 3.6. Isolation of a transient outward current based on voltage-dependence of inactivation. A. Total outward currents elicited by 1-sec command potentials from -72 to +38 mV in 10 mV increments following a 500-msec conditioning pre-pulse to -132 mV (upper panel) and -52 mV (middle panel) from a holding potential of -82 mV. Lower panel: is a point-by-point subtraction of upper and middle traces, revealing a transient outward current. B. Current-voltage relationship of the peak outward currents elicited by the command potentials indicated following conditioning pre-pulses to -132 mV (*filled circles*) or -52 mV (*open circles*) and the derived outward current (*filled diamonds*; mean ± SEM, n = 4). All currents were normalized to the maximum outward current elicited during the various command potentials following a conditioning pre-pulse to -132 mV. All recordings were carried out from September to January.

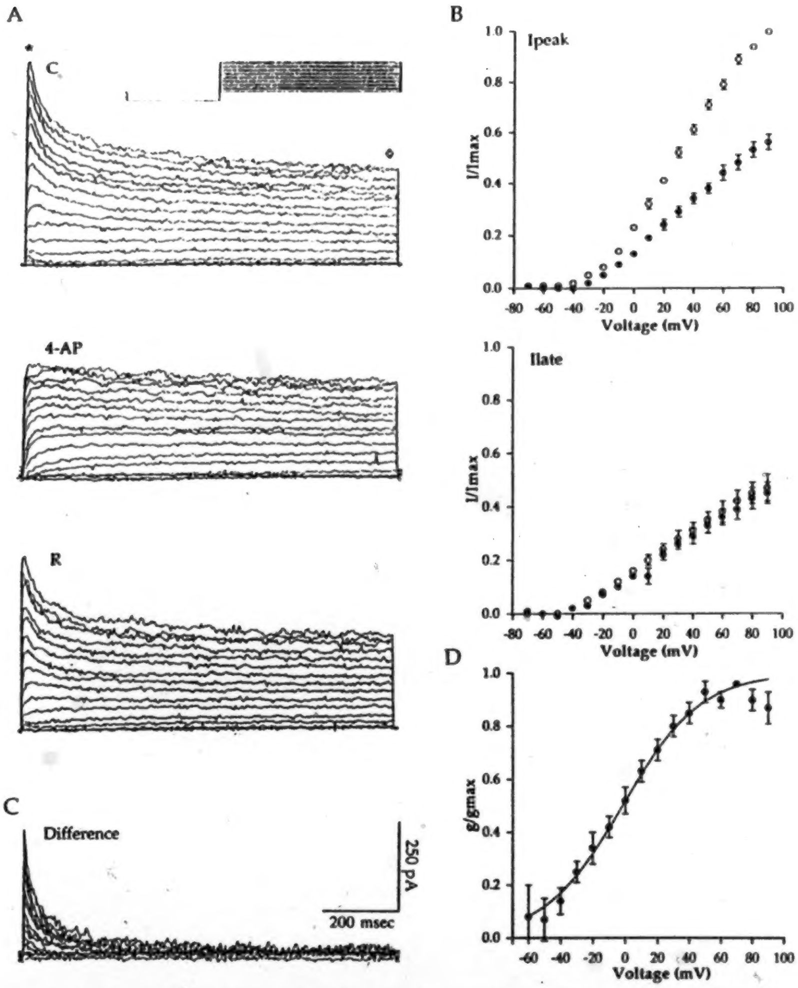

Fig. 3.7. Effects of 4-AP on voltage-dependent K^+ currents in identified goldfish gonadotrophs Currents were elicited by 1-sec depolarizing voltage steps from -82 mV to +78 mV following a 500-msec conditioning pre-pulse to -132 mV (holding potential = -82 mV). A. Representative current traces before (C, control), during (4-AP) and after (R, recovery) the application of 2 mM 4-AP are shown. B. Current-voltage relation of average peak (*asterisk*; 0 - 20 msec) and sustained (*open diamond*; 980 - 1000 msec) outward currents before (*open circles*) and during (*filled circles*) the application of 2 mM 4-AP (mean ± SEM,n = 6). Currents were normalized to the maximal outward current. C. The 4-AP-sensitive current was obtained by point-by-point subtraction of the currents in A (C - 4-AP). D. Activation curve for the 4-AP-sensitive current. The solid line is a fitted Boltzmann relation of the form $g/g_{max}=g_{max}/\{1+\exp[(E-E_{1/2})/k]\}$. Conductances in D were normalized to the maximum conductance. All recordings were carried out from September to January.

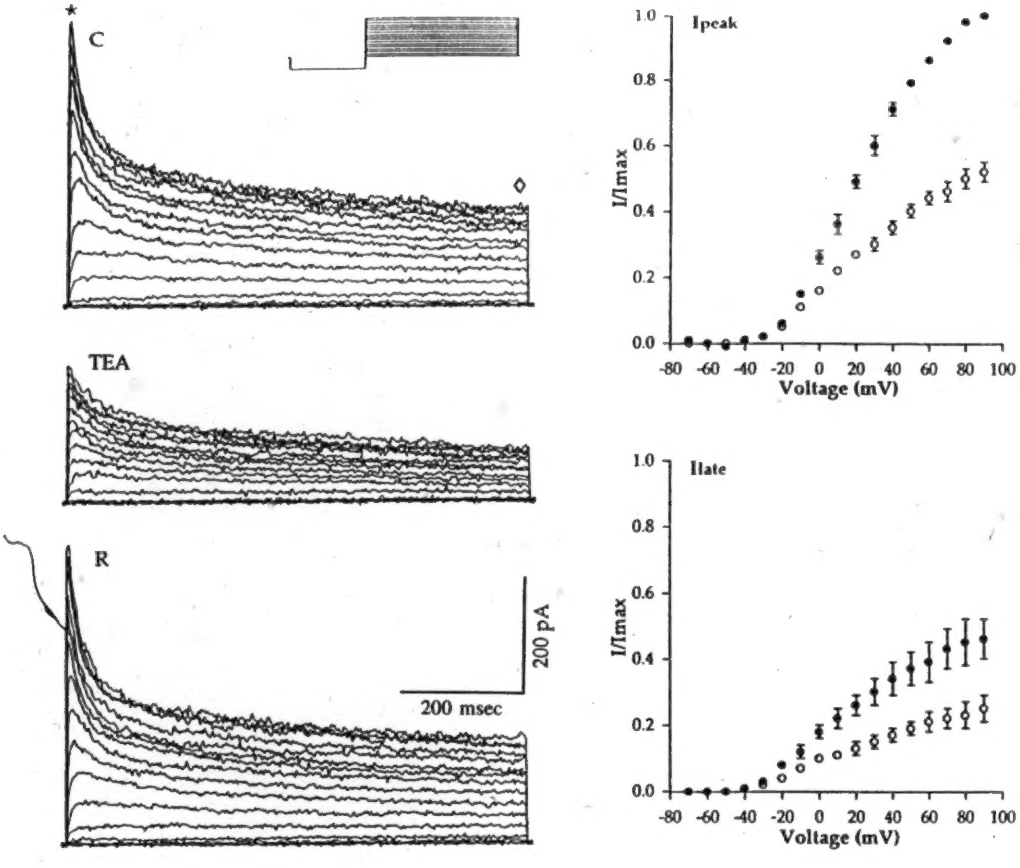

Fig. 3.8. Effects of TEA on voltage-dependent K+ currents in identified goldfish gonadotrophs. A. Currents were elicited by 1-sec depolarizing voltage steps from -82 mV to +78 mV following a 500-msec conditioning prepulse to -132 mV (holding potential = -82 mV). Representative current traces of control (C), 5 mM TEA-treated (TEA) and recovered (R) K+ currents from the same cell are shown. B. The current-voltage relation of the average peak (*asterisk*) K+ currents in the absence (*filled circles*) and presence (*open circles*) of 5 mM TEA (mean ± SEM, *n* = 6). C. The current-voltage relationship of the average sustained (*open diamond*) K+ current in the absence (*filled circles*) and presence of 5 mM TEA (*open circles*; mean ± SEM, *n* = 6). All recordings were carried out from September to January.

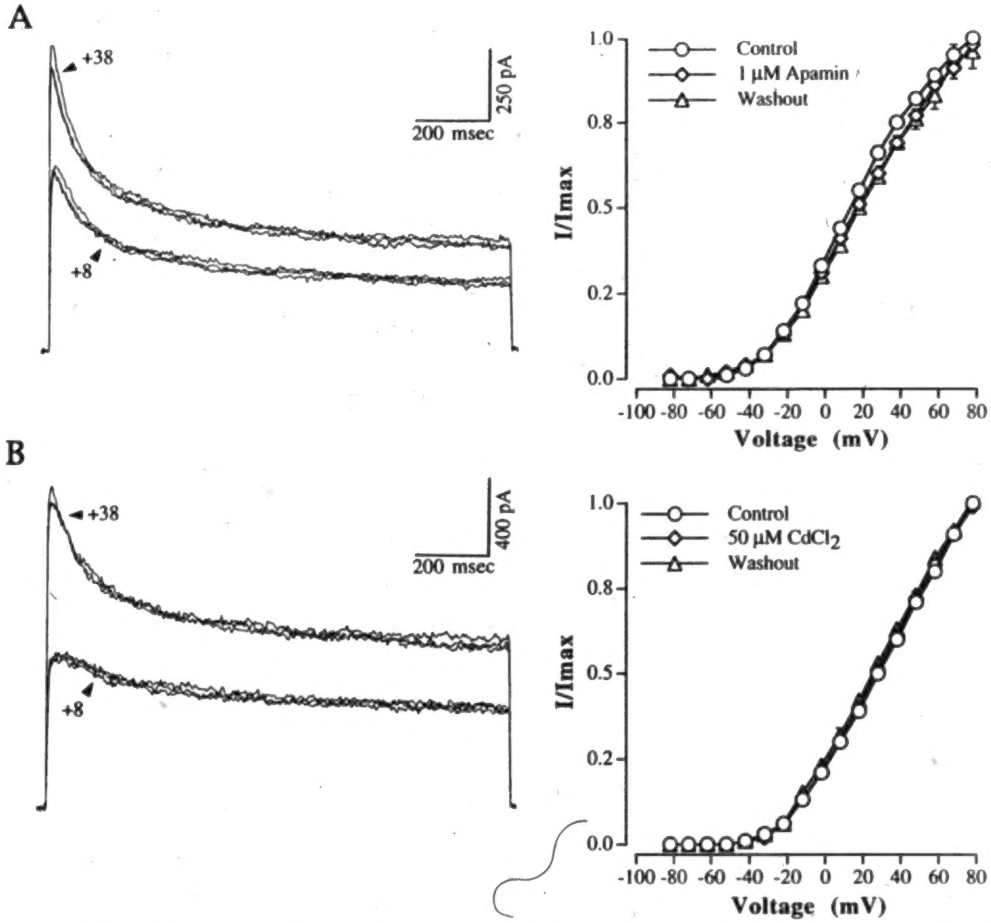

Fig. 3.9. Absence of calcium-activated K$^+$ currents in identified goldfish gonadotrophs. All currents were elicited by 1-sec depolarizing voltage steps from -82 mV to +78 mV following a 500 msec conditioning pre-pulse to -132 mV (holding potential = -82 mV). A. Left panel: supperimposed current traces elicited by a 1-sec voltage-step from -132 mV to +38 mV before (CTR), during (Apamin) and after (R) the application of 1 µM apamin. Right panel: the current-voltage relation of the average peak K$^+$ currents before (*filled circles*), durring (*open circles*) and after (*open squares*) the application of 1 µM apamin (mean ± SEM, *n* = 5). B. Left panel: supperimposed current traces elicited by a 1-sec voltage-step from -132 mV to +38 mV before (CTR), during (Cd^{2+}) and after (R) the application of 50 µM CdCl$_2$. Right panel: the current-voltage relation of peak K$^+$ currents before (*filled circles*), durring (*open circles*) and after (*open squares*) the application of 50 µM CdCl$_2$ (mean ± SEM, *n* = 4). All recordings were performed with 5 mM extracellular Ca^{2+} in the external medium and were carried out from October to December.

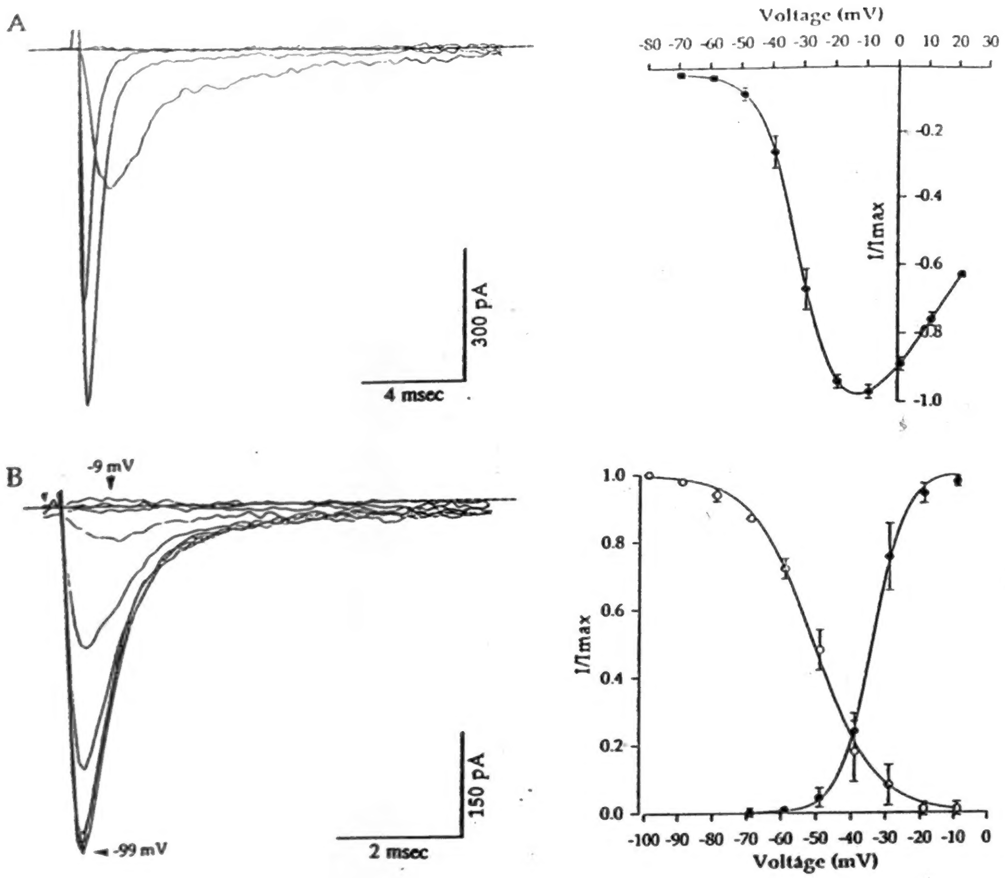

Fig. 3.10. Transient voltage-dependent Na^+ current in identified goldfish gonadotrophs. A. The current-voltage relationship of the Na^+ current was obtained by holding the cells at -79 mV and then stepping for 100 msec to command potentials between -69 and +21 mV, in 10 mV increments (mean ± SEM, n = 9). Shown at left are representative current traces elicited during command potentials to -69, -29, -9 and +11 mV. B. Steady-state inactivation and activation curves for the voltage-dependent Na^+ current (mean ± SEM, n = 6). Inactivation curves (*open circles*) were generated from prepulse experiments where the membrane potential was stepped to between -99 and -9 mV for 100 msec prior to stepping to a test potential of -9 mV. Activation curves (*filled circles*) were generated from the same cells. Shown at left are representative traces of the remaining currents elicited by a test pulse to -9 mV following prepulse potentials between -99 and -9 mV. The solid line for the steady state inactivation curve is a fitted Boltzmann relation (equation 1), where $E_{1/2}$ = -50.8 mV and k = 8.8. All currents were normalized to the maximal inward current. All recordings were carried out from July to August.

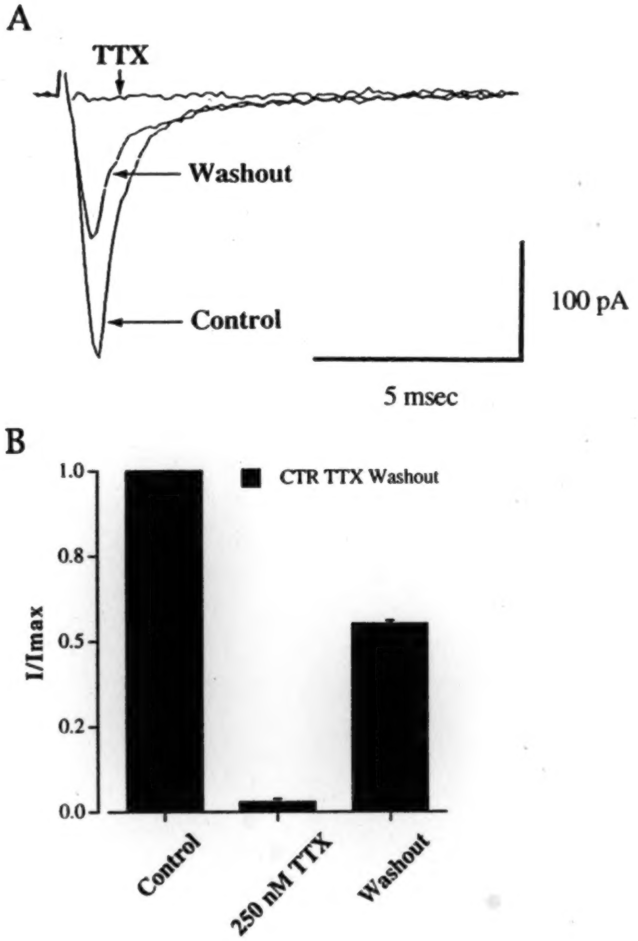

Fig. 3.11. Tetrodotoxin sensitivity of Na^+ currents in identified goldfish gonadotrophs. A. Representative Na^+ current trace under voltage-clamp recording conditions in the presence or absence of 250 nM TTX. B. The voltage-dependent Na^+ current normalized to the percentage of the peak Na^+ current before, during and after the application of TTX (mean ± SEM). *Asterisk* denotes significant difference form control values; *double asterisks* denotes significant difference from control and TTX ($P < 0.05$, ANOVA, followed by Fisher's LSD test, $n = 4$)

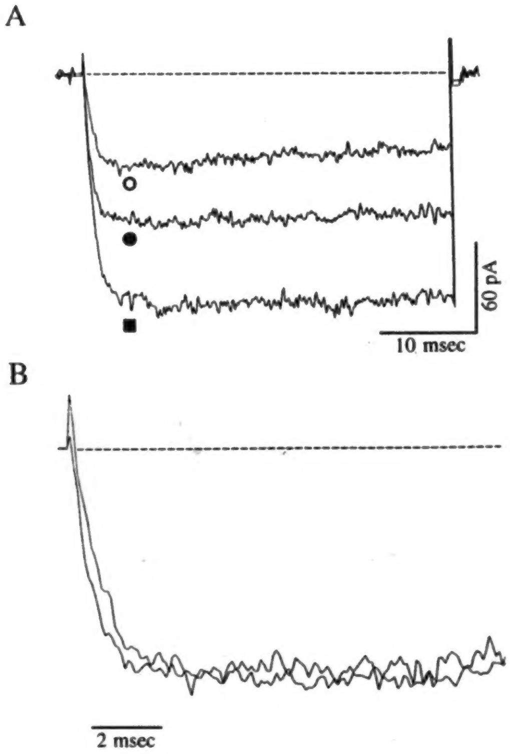

Fig. 3.12. Whole-cell perforated-patch recordings of Ca^{2+} and Ba^{2+} currents through voltage-dependent Ca^{2+} channels in identified goldfish gonadotrophs. A. Representative current traces elicited during a 40 msec voltage-step to -12 mV (holding potential = -92 mV) in the presence of 10 mM Ca^{2+} (open circle), 10 mM Ba^{2+} (filled circle) or 20 mM Ba^{2+} (filled square). B. In order to compare the activation kinetics between Ca^{2+} and Ba^{2+} currents through voltage-dependent Ca^{2+} channels, currents carried by either 10 mM Ca^{2+} or 10 mM Ba^{2+} were normalized to their respective maximal inward current and superimposed. Currents were elicited during a 40-msec voltage-step to -12 mV from a holding potential of -92 mV. All current recordings were carried out from October to November.

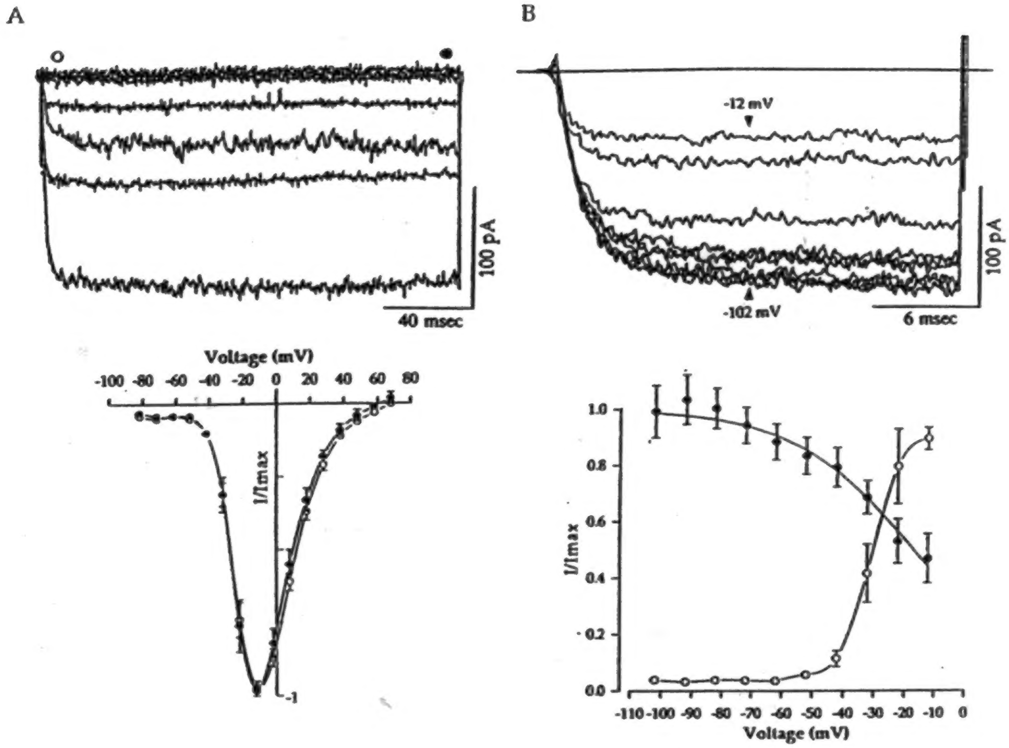

Fig. 3.13. Whole-cell recordings of voltage-dependent Ba^{2+} currents through Ca^{2+} channels in identified goldfish gonadotrophs. A. Upper panel: representative current traces elicited by a 200 msec command potential to -72, -52, -42, -12, +8, +28, +48 and +68 mV from a holding potential of -92 mV. Lower panel: current-voltage relation of the peak (*open circles*; 0 - 20 msec) and sustained (*filled circles*; 180 - 200 msec) Ba^{2+} currents (mean ± SEM,n = 9). B. Activation and steady-state inactivation properties of Ba^{2+} currents. Upper panel: representative current traces elicited by a 20 msec command potential of -12 mV following a 5-sec conditioning pre-pulse from -102 to -12 mV. Lower panel: activation (*open circles*) and steady-state inactivation (*filled circles*) curves of Ba^{2+} current (mean ± SEM,n = 6). The solid line for the steady state inactivation curve is a fitted Boltzmann relation (equation 1), where $E_{1/2}$ = -16.7 mV and k = 20. Currents were normalized to the maximal inward current. All recordings were carried out from October to December.

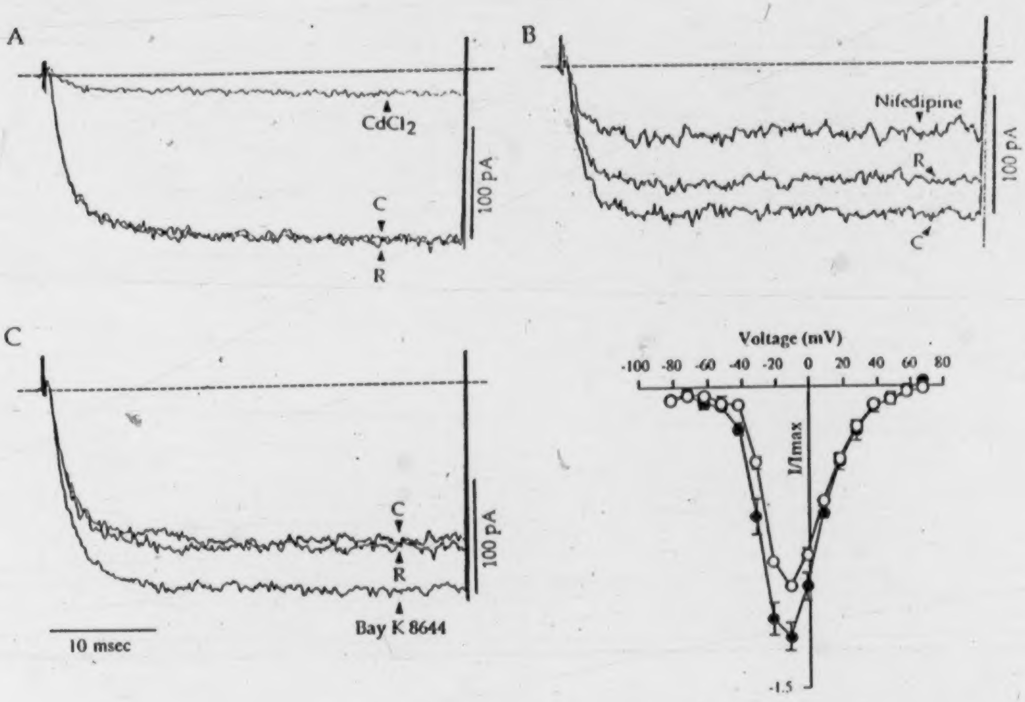

Fig. 3.14. Effects of $CdCl_2$, nifedipine and S(-)-Bay K 8644 on Ba^{2+} currents through Ca^{2+} channels in identified goldfish gonadotrophs. A. Currents elicited during a 40 msec step to -12 mV before (C), during ($CdCl_2$) and after (R) the application of 50 µM $CdCl_2$ (holding potential = -92 mV). B. Currents elicited during a 40 msec step to -12 mV before (C), during (Nifedipine) and after (R) the application of 10 µM nifedipine (holding potential = -80 mV). C. Left panel: currents elicited during a 40 msec step to -12 mV before (C), during (Bay K 8644) and after (R) the application of 1 µM S(-)-Bay K 8644 (holding potential = -92 mV). Right panel: current-voltage relation of the Ba^{2+} current before (*open circles*) and after (*filled circles*) the application of S(-)-Bay K 8644 (mean ± SEM, n = 4). Currents were normalized to maximal inward current. All recordings were carried out between October and December.

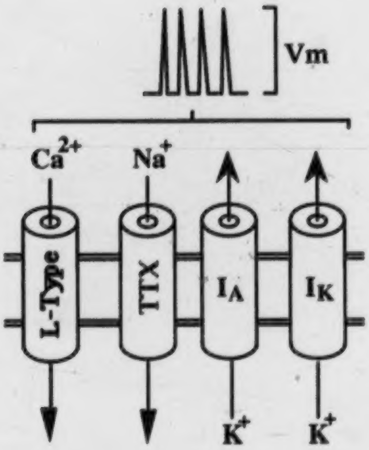

Fig. 3.15. Electrical membrane properties and voltage-dependent currents observed in identified goldfish gonadotrophs.

Chapter 4

INVOLVEMENT OF EXTRACELLULAR SODIUM IN AGONIST-INDUCED GONADOTROPIN RELEASE FROM GOLDFISH GONADOTROPHS[†]

INTRODUCTION

Ionic flux across the plasma membrane through voltage-dependent and -independent ionic channels is important in the regulation of many cellular functions, including hormone release. In mammals, LH release is stimulated by the hypothalamic decapeptide, mGnRH (Chang and Jobin, 1994a). Activation of mGnRH-receptors in the plasma membrane results in the oscillatory release of intracellular Ca^{2+} from $InsP_3$-sensitive stores, which leads to the generation of periodic membrane hyperpolarizations followed by one or more regenerative action potentials (Hille *et al.*, 1994, 1995; Stojilkovic *et al.*, 1994), as well as exocytosis (Tse *et al.*, 1993; Hille *et al.*, 1994). These mGnRH-induced membrane potential changes are mediated by multiple ionic channels, including voltage-dependent Na^+ and Ca^{2+} channels, as well as Ca^{2+}-activated, apamin-sensitive K^+ channels (Stojilkovic *et al.*, 1994; Hille *et al.*, 1995). In addition, GnRH action has also been linked to the activation of transport proteins such as Na^+/H^+ exchangers (McArdle *et al.*, 1991; Bouali-Benazzouz *et al.*, 1993).

As in mammals, application of GnRH results in a rise in $[Ca^{2+}]_i$ in teleostean pituitary cells (Jobin and Chang, 1992b; Kah *et al.*, 1992; Chang *et al.*,

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[†] A version of this chapter has been published: Van Goor, F., Goldberg, J. I., and Chang, J. P. (1996). Endocrinology. 137, 2859-2871.

1993, 1996; Rebers *et al.*, 1995). In goldfish, the two endogenous GnRHs, sGnRH and cGnRH-II, are released directly into the pituitary and bind to the same class of high-affinity, low-capacity receptors to stimulate maturational gonadotropin (GTH-II, the functional equivalent of LH; Van Der Kraak *et al.*, 1992) release (Peter *et al.*, 1991; Chang *et al.*, 1993). The GTH-II-releasing action of the two native GnRHs is mediated by a Ca^{2+}/PKC-dependent signaling pathway (Chang *et al.*, 1993). Extracellular Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channels and the resulting increase in $[Ca^{2+}]_i$ is believed to play a central role in GTH-II exocytosis (Jobin and Chang, 1992a; Chang *et al.*, 1993, 1996). Pharmacological studies have implicated PKC in mediating the GnRH-induced activation of dihydropyridine-sensitive Ca^{2+} channels that leads to Ca^{2+} entry and the subsequent release of GTH-II (Chang *et al.*, 1991a; Jobin and Chang, 1992b; Jobin *et al.*, 1996). Recently, the voltage-dependent and pharmacological properties of the Ca^{2+} channels in identified goldfish gonadotrophs were characterized using whole-cell, voltage-clamp recordings (Chapter 3). These Ca^{2+} channels were similar to the high-voltage activated and inactivation-resistant channels, or "L-type" channels, identified in other vertebrate gonadotrophs (Tse and Hille, 1993). Although the participation of extracellular Ca^{2+} in the regulation of GTH-II release is well understood, the role of other extracellular ions, such as Na^+, is not well defined in teleosts or other lower vertebrates.

Actions of extracellular Na^+ at the plasma membrane mediate a diverse array of cellular functions, including the regulation of membrane excitability (Hodgkin and Huxley, 1952) and cytosolic pH (Noël and Pouysségur, 1995). For example, in male rat gonadotrophs, voltage-dependent Na^+ channels contribute to the up-stroke of the action potential generated by GnRH (Tse and Hille, 1993). Also, the Na^+/H^+ exchanger may play an important role in regulating cellular pH during GnRH-stimulated LH release from the rat pituitary (McArdle *et al.*, 1991; Bouali-Benazzouz *et al.*, 1993). In these and other cells, the increase in Na^+/H^+ exchanger activity is a consequence of agonist-induced mobilization of intracellular Ca^{2+} and PKC activation (Bouali-Benazzouz *et al.*, 1993; Noël and Pouysségur, 1995). Sodium has also been demonstrated to indirectly modulate stimulus-secretion coupling by regulating Ca^{2+} dynamics (Zucker, 1993). Furthermore, it may even exert a direct, Ca^{2+}-independent effect on stimulus-secretion coupling in some cells (Nordmann *et al.*, 1992). Therefore, understanding the role of extracellular Na^+ in mediating GnRH action is important in elucidating the cellular events underlying hormone secretion.

In this study, we investigated the involvement of extracellular Na^+ in the regulation of GTH-II release from goldfish gonadotrophs. In perifusion studies with dispersed goldfish pituitary cells, we examined the effects of Na^+-depleted medium and a Na^+ channel inhibitor on GnRH-stimulated GTH-II secretion. The effects of increased Na^+ influx on GTH-II release was similarly investigated using a Na^+ channel modulator, veratridine. In addition, the direct action of GnRH on voltage-dependent Na^+ channels was investigated in electrophysiological studies of identified goldfish gonadotrophs. To investigate the possible involvement of Na^+/H^+ exchangers, the actions of the Na^+/H^+ exchanger inhibitors, amiloride and 5-(N,N-dimethyl)-amiloride (DMA), on GnRH- and PKC-stimulated GTH-II release were monitored in static incubation experiments. The effects of Na^+-depletion on PKC-stimulated GTH-II release were similarly confirmed in static incubation studies. In general, GnRH-stimulated GTH-II release is dependent on extracellular Na^+ and involves the activity of amiloride-sensitive Na^+/H^+ exchangers, but not voltage-dependent Na^+ channels.

MATERIALS AND METHODS

Animals

Common goldfish (8-13 cm in body length) were purchased from Grassyforks Fisheries (Martinsville, IN) or Ozark Fisheries (Stoutland, MO) and held in flow-through aquaria (1800 l) under the conditions described previously in Chapter 3. In the present study, experiments were repeated using fish of both sexes from different stages of the seasonal gonadal cycle. Athough basal GTH-II release fluctuated throughout the year, the results were similar regardless of the gonadal status of the pituitary donor fish. Therefore, results from replicate experiments performed throughout the year were pooled. Nevertheless, in order to facilitate future comparisons, the gonadal stages for each set of experiments are reported in the figure legends.

Cell culture and static incubation studies of GTH-II responses

Fish were anesthetized in 0.05% tricaine methanesulfonate prior to decapitation. Pituitaries from both male and female goldfish were removed and pituitary cells dispersed using a controlled trypsin/DNAse treatment procedure (Chang *et al.*, 1990a). The cells were resuspended in plating medium (Medium 199 with Earles' salts, 1% horse serum, 25 mM HEPES, 2.2 g/l $NaHCO_3$, 100 000 U/l penicillin and 100 mg/l streptomycin, pH adjusted to 7.2 with NaOH; Gibco) and cultured overnight in 24-well culture plates (0.25×10^6 cells/ml/well) under 5% CO_2, saturated humidity, and at 28 °C. Prior to each experiment, cells were washed with testing medium (Medium 199 with Hank's salts (Gibco), 2.2 g/l $NaHCO_3$, 25 mM HEPES, 100 000 U/l penicillin, 100 mg/l streptomycin, 0.1% BSA; pH adjusted to 7.2 with NaOH) and allowed to rest for 1 hr under the conditions described above. The cells were subsequently incubated under similar conditions in testing medium supplemented with various secretagogues and inhibitors for 2 hr, after which the medium was removed and stored at -20 °C. Gonadotropin content was measured using a RIA specific for GTH-II (maturational GTH), as previously described (Peter *et al.*, 1984; Van Der Kraak *et al.*, 1992). All treatments were tested in triplicate or quadruplicate and each experiment was repeated a minimum of three times. Results from replicate experiments were expressed as a percentage of basal release (unstimulated control) and pooled data (mean ± SEM) are presented. Statistical analyses were performed using analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test or unpaired t test. Differences between groups were considered significant when $P < 0.05$.

Cell column perifusion studies of GTH-II responses

For the measurement of acute hormone release responses to various stimulators and inhibitors, cell column perifusion studies were performed as previously described (Chang *et al.*, 1990b). Briefly, dispersed goldfish pituitary cells were cultured for 16 to 48 hr on preswollen Cytodex-I beads under the conditions described above. Prior to each experiment, the cells were loaded onto perifusion columns (2 x 10^6 cells/column) and perifused with testing medium for 4 hr at a rate of 15 ml/hr, after which a relatively stable basal secretion rate was established. Experiments began with the collection of 5-min fractions of perifusate. Perifusates were kept frozen at -20 °C until their GTH-II contents could be measured using a GTH-II specific RIA (Peter *et al.*, 1984; Van Der Kraak *et al.*, 1992). GTH-II content in individual columns was normalized as a percentage of the average pretreatment values obtained in the first four fractions collected (% Pretreatment). This data transformation was done to account for fluctuations in basal hormone release observed throughout the year and to allow pooling of the data from separate columns of the same experiment, without distorting the profile of hormone release during the course of perifusion. The hormone responses were quantified by determining the net change in GTH-II levels (*i.e.* area under the curve) and expressed as a percentage of pretreatment values as previously described (Wong *et al.*, 1992). All values in the text and figures are reported as mean ± SEM. Differences between groups were considered to be significant when $P < 0.05$ using ANOVA, followed by LSD difference test or paired *t* test.

For experiments in which extracellular ion concentrations were modified, the external bath solution contained either modified testing medium (M-199 with Hank's salts prepared without the addition of NaCl or $CaCl_2$; courtesy of Dr. S.S. Stojilkovic, ERRB, NICHD, National Institutes of Health, Bethesda, MD; Na^+ and Ca^{2+} levels were adjusted as required, see below) or modified Hank's balanced salt solution, consisting of (in mM): 5.4 KCl, 0.4 KH_2PO_4, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 0.3 $NaHPO_4$, 4.2 $NaHCO_3$, 5.5 glucose, 15 HEPES. Hormone release responses in modified testing medium and Hank's balanced salt solution were similar and therefore pooled. Under control conditions, the external medium contained 136 mM NaCl and 1.26 mM $CaCl_2$. For experiments using Na^+-depleted medium, NaCl was replaced with equimolar choline-chloride. In experiments using Ca^{2+}-free media, $CaCl_2$ was omitted and 0.1 mM EGTA was added to remove any residual Ca^{2+}. All solutions were supplemented with 2.2 g/l $NaHCO_3$, 25 mM HEPES, 100 000 U/l penicillin, 100 mg/l streptomycin, 0.1% BSA and pH was adjusted to 7.2 with TrisCl.

Electrophysiological Recordings

For electrophysiological studies, dispersed goldfish pituitary cells (0.25 x 10^6 cells/ml) were plated for 16 hr on poly-L-lysine-coated (0.01 mg/ml) glass-bottom petri dishes under the conditions described above. Gonadotrophs were identified on the basis of their unique morphological characteristics as previously described (Chapter 2). Whole-cell, voltage-clamp recordings (Hamill *et al.*, 1981) were performed at room temperature (18 °C to 20 °C) using nystatin-perforated patch recording techniques (Korn and Horn, 1989). Voltage-clamp recordings were carried out using a Dagan 3900 (Minneapolis, MN) integrating patch-clamp amplifier and were low-pass filtered at 2 KHz using a four-pole bessel filter. Patch electrodes, fabricated from borosilicate glass (1.5 mm OD; World Precision Instruments, Sarasota, FL) using a Flaming Brown horizontal puller (P-87; Sutter Instruments, Novato, CA), were heat polished to a final tip resistance of 3 to 5 MΩ and then coated with dental wax to reduce pipette capacitance. A nystatin stock solution (50 mg/ml) was prepared in dimethyl sulfoxide (DMSO) and stored for up to 1 week at -20 °C. Just prior to use, the nystatin stock solution was diluted in pipette solution, containing (in mM): 70 Cs-glutamate, 70 CsCl, 2 $MgCl_2$, 10 HEPES (pH adjusted to 7.2 with TrisCl) and sonicated for 30 sec to yield a final nystatin concentration of 160 µg/ml. Pipette tips were briefly immersed in nystatin-free solution, and then backfilled with the same solution containing nystatin. After obtaining a GΩ-seal and neutralizing pipette capacitance, the pipette potential was set to -70 mV and a 10 mV hyperpolarizing voltage step was given to monitor access resistance. An average series resistance of 26.3 ± 1.8 MΩ (n = 10) was reached 10 min following the formation of a GΩ-seal and remained stable for up to one hour. Series resistance (R_s) was calculated from the relation $R_s = \tau/C_m$, where τ is the decay time constant of the capacitive current transient following a 10 mV hyperpolarizing voltage pulse ($\tau = 0.14 \pm 0.01$ msec; mean ± SEM; $n = 10$) and C_m is the cell capacitance. The cell capacitance was determined by integrating the capacity current transient during a 10 mV hyperpolarizing voltage pulse (Vm) and applying the relation $Cm = q/Vm$. Cell capacitance was in the range of 4 - 7 pF (5.2 ± 0.4 pF; $n = 10$). Series resistance compensation was optimized and usually ranged between 10 and 18 mΩ. Current records were corrected for linear leakage and capacitance using a P/-4 procedure (Bezanilla and Armstrong, 1977). Pulse generation, and data acquisition were carried out using an AT 486 2DX compatible computer equipped with a digidata 1200 interface in conjunction with pCLAMP programs (Axon Instruments, Foster City, CA). The effects of all drug applications on Na^+ or Ca^{2+} currents were continuously monitored by giving a 40 msec voltage-step to 0 mV from a holding potential of -70 mV, every 5-sec. Current-voltage relationships and steady-state inactivation curves were generated only after the effect of the drug application had stabilized.

For recordings of isolated Na^+ currents, the external solution contained (in mM): 136 NaCl, 1.26 $CaCl_2$, 2.5 KCl, 1 $MgCl_2$, 0.5 $CdCl_2$, 10 TEA-Cl, 8 glucose, and 10 HEPES (pH adjusted to 7.2 with NaOH). In experiments on isolated Ca^{2+} currents, Ba^{2+} was used as the charge carrier through Ca^{2+} channels. For Ba^{2+} current recordings, the external solution contained (in mM) 120 N-methyl-D-glucamine-Cl, 30 $BaCl_2$, 1.0 $MgCl_2$, 2.5 KCl, 8 glucose, 10 HEPES (pH adjusted to 7.2 with HCl). Barium was used as the charge carrier instead of Ca^{2+} because it permeates more readily than Ca^{2+} through Ca^{2+} channels, it is relatively impermeant through Na^+ channels and it blocks outward K^+ currents (Bean, 1992). A solid Ag/AgCl reference electrode was connected to the bath via a 3 M KCl agar bridge. The bath contained < 200 µl of saline and was continuously perifused at a rate of 2 ml/min using a gravity driven superfusion system. The outflow was placed near the cell, resulting in complete solution exchange around the cell within 2 sec.

Data analysis was performed using an AT 486 2DX compatible computer in conjunction with Clampfit (Axon Instruments, Foster City, CA). For steady-state inactivation curves, the data were fit with the Boltzmann relation;

$$I/I_{max} = I_{max}/\{1+\exp[(E-E_{1/2})/k]\} \qquad \text{(equation 1)}$$

where I_{max} = the maximal current, E = test potential, $E_{1/2}$ = the potential of 50 % maximal current and k = slope factor. Exponential fits for the determination of τ were performed using Clampfit. All reported membrane potentials for Na^+ currents were corrected for a liquid junction potential between the pipette and bath solutions of 10 mV. The junction potential for Ba^{2+} current recordings was 2.5 mV and was not corrected for. All liquid junction potentials were calculated using the JPCalc software program (Barry, 1994). All values in the text are reported as mean ± SEM. Differences between groups were considered to be significant when $P < 0.05$ using paired t test.

Drugs

To make stock solutions, sGnRH and cGnRH-II (Peninsula Laboratories, Belmont, CA), as well as amiloride and DMA (Sigma, St Louis, MO), were dissolved in distilled de-ionized water. Tetrodotoxin (TTX), veratridine and phorbol 12-myristate 13-acetate (TPA; Research Biochemicals Incorporated, Natick, MA) were dissolved in ethanol. Aliquots of stock solutions were stored at -20 C until use, when they were diluted to final concentrations in saline. The highest concentration of ethanol or DMSO was < 0.1%, which had no effect on either basal hormone release or ionic currents. TEA (Sigma, St Louis, MO) was prepared in saline immediately prior to use.

RESULTS

Extracellular Na^+ dependence of agonist-induced GTH-II release

The involvement of extracellular Na^+ in GnRH-stimulated GTH-II release was examined in perifusion experiments on dispersed goldfish pituitary cells. As in previous perifusion studies (Chang *et al.*, 1990b), the GTH-II release responses to three sequential pulses of either sGnRH or cGnRH-II, applied at 65 min intervals, were similar over the duration of the experiment in control (Na^+-containing medium) columns. Net GTH-II responses in three sequential pulses of 100 nM sGnRH were: 53.6 ± 6.8, 57.1 ± 14.7 and 52.8 ± 14.8%; the net GTH-II responses in three sequential pulses of 100 nM cGnRH-II were: 87.9 ± 10.9, 77.15 ± 32.1 and 48.4 ± 8.6% ($P > 0.05$, ANOVA followed by LSD test). Depletion of extracellular Na^+ by replacing NaCl with equimolar choline-chloride reduced the GTH-II-release response to 100 nM sGnRH; replacement of Na^+ to the medium completely restored the GTH-II response (Fig. 4.1A). Similarly, treatment with Na^+-depleted medium reversibly reduced 100 nM cGnRH-II-stimulated GTH-II release (Fig. 4.1B). These results indicate that extracellular Na^+ is involved in GnRH-stimulated GTH-II release.

In goldfish, activation of PKC has been implicated in mediating sGnRH- and cGnRH-II-stimulated GTH-II release (Chang *et al.*, 1993, 1996). Therefore, the involvement of extracellular Na^+ in PKC-stimulated GTH-II release was also monitored in static incubation studies. The PKC activator, TPA, stimulated GTH-II release in a dose-dependent manner (Table 4.1). Incubation with extracellular Na^+-depleted medium significantly reduced both 1 and 10 nM TPA-stimulated GTH-II release (Table 4.1). These results indicate that extracellular Na^+ is involved in PKC-stimulated GTH-II release.

In addition, these results are consistent with the hypothesis that PKC is involved in GnRH-stimulated GTH-II release from the goldfish pituitary.

Voltage-dependent Na^+ channels are not involved in GnRH action

In Chapter 3, voltage-dependent Na^+ currents were characterized in identified goldfish gonadotrophs. To test the possible involvement of voltage-dependent Na^+ currents during GnRH-stimulated GTH-II release, we used both electrophysiological techniques and perifusion studies. The effects of sGnRH and cGnRH-II on voltage-dependent Na^+ currents were monitored with nystatin-perforated-patch voltage-clamp recordings. The use of perforated-patch recordings prevents the diffusion of cytoplasmic constituents, which may participate in the mediation of GnRH action, into the patch pipette (Korn and Horn, 1989). In identified goldfish gonadotrophs, fast-activating and fast-inactivating Na^+ currents were detected at command potentials more depolarized than -60 mV and reached a maximum amplitude between -20 and 0 mV (Fig. 4.2A). Application of sGnRH (100 nM; $n = 3$) and cGnRH-II (100 nM; $n = 4$) did not affect the current-voltage relationship of the voltage-dependent Na^+ current. In addition, neither sGnRH nor cGnRH-II affected the Na^+ current amplitude in response to a 40-msec voltage-pulse to -10 mV from a holding potential of -80 mV. The peak Na^+ currents measured before, during and after the application of 100 nM sGnRH were 551.8 ± 79.4 pA, 562.3 ± 82.4 pA and 578.1 ± 93.1 pA, respectively ($P > 0.05$; $n = 10$). The peak Na^+ currents measured before, during and after the application of 100 nM cGnRH-II were 478.2 ± 61.2 pA, cGnRH-II 483.8 ± 87.6 pA 505.5 ± 87.6 pA, respectively ($P > 0.05$; $n = 13$).

To determine if GnRH affects the steady-state inactivation properties of the voltage-dependent Na^+ current, a two-pulse protocol was used (Bezanilla and Armstrong, 1977). Cells held at -80 mV were subjected to pre-pulse potentials ranging from - 100 to +10 mV for 100 msec, after which a 40 msec test pulse to -10 mV was applied (Fig 4.2B). The normalized test current was plotted against prepulse potentials and the resulting curve was fit with a Boltzmann relation (equation 1). Neither sGnRH nor cGnRH-II altered the mid-point ($E_{1/2}$) or the slope factor (k) of the Na^+ current steady-state inactivation curve (Table 2; Fig 2B). These results indicate that GnRH does not affect the steady-state inactivation properties of the voltage-dependent Na^+ current.

The voltage-dependent Na^+ channel blocker, TTX, has been demonstrated to block Na^+ currents in identified goldfish gonadotrophs (Chapter 3), as well as in other vertebrate gonadotrophs (Mason and Sikdar, 1988; Tse and Hille, 1993). Therefore, we used TTX to further investigate the involvement of voltage-dependent Na^+ channels during GnRH-stimulated GTH-II release. In perifused dispersed pituitary cells, addition of 1 μM TTX did not affect basal release of GTH-II (control 11.2 ± 1.9 ng/ml/2 x 10^6 cells *vs.* TTX 10.8 ± 1.9 ng/ml/2x10^6 cells; $P > 0.05$, paired t test; $n = 12$), nor the GTH-II-release responses to either 100 nM sGnRH or 100 nM cGnRH-II (Fig. 4.3; $P > 0.05$, paired t test, control *vs.* TTX-treated, TTX-treated *vs.* washout). Taken together, the results from both hormone release and electrophysiological studies suggest that voltage-dependent, TTX-sensitive Na^+ channels are not involved in mediating either basal or GnRH-stimulated GTH-II release.

Na^+ influx and GTH-II release

Veratridine promotes Na^+ flux through voltage-dependent Na^+ channels, resulting in an increase in cytosolic Na^+ concentration and membrane depolarization (Catterall and Nirenberg, 1973). To test the possible involvement of extracellular Na^+ influx in GTH-II release, the hormone release response to veratridine was monitored in perifusion studies. In all columns, control pulses of cGnRH-II (100 nM) in normal medium were given 70 min before and 70 min after the application of veratridine. Twenty min before the application of veratridine (at time = 90 min), the medium was switched to one of three media: 1) normal; 2) Ca^{2+}-free (M-199 without Ca^{2+} salts but with the addition of 0.1 mM EGTA); or 3) Na^+-depleted, Ca^{2+} free medium. When measured in normal test medium, GTH-II release was stimulated by 183.6 ± 32.2% ($n = 8$) over basal levels after addition of 100 µM veratridine (Fig. 4.4). As the depolarizing action of veratridine would induce the opening of voltage-dependent Ca^{2+} channels, we tested whether veratridine-stimulated GTH-II release was dependent on extracellular Ca^{2+}. In Ca^{2+}-free medium, 100 µM veratridine was still able to significantly stimulate GTH-II release by 71.1 ± 14.8% ($n = 8$) over basal levels. Thus, veratridine can stimulate release in the absence of extracellular Ca^{2+} influx through voltage-dependent ion channels. However, the GTH-II release response to veratridine in Na^+-depleted, Ca^{2+}-free medium was greatly reduced compared to normal and Ca^{2+}-free conditions (17.3 ± 5.5% above basal; $n = 8$). In all columns, GTH-II release responses to control pulses of 100 nM cGnRH-II in normal medium given prior to and after the application of veratridine were similar (data not shown). Therefore, prior treatment with Ca^{2+}-free or Na^+-depleted, Ca^{2+}-free medium did not affect subsequent GTH-II release responses to cGnRH-II.

Involvement of Na^+/H^+ exchange in GnRH action

Removal of extracellular Na^+ may influence the activity of plasma membrane proteins such as Na^+/H^+ exchangers. To investigate the possible involvement of Na^+/H^+ exchangers during GnRH-stimulated GTH-II release, the effects of amiloride and DMA, which have been reported to selectively block the Na^+/H^+ exchanger (Noël and Pouysségur, 1995), were monitored in static-incubation studies. Both amiloride (10 and 100 μM) and DMA (0.1 to 100 μM) reduced 100 nM sGnRH-stimulated GTH-II release (Fig. 4.5A). Similarly, amiloride (10 μM and 100 μM) and DMA (10 μM and 100 μM) decreased 100 nM cGnRH-II-stimulated GTH-II release (Fig. 4.5B). In general, basal GTH-II release was not affected by 2-hr treatments with amiloride or DMA.

The above results suggest that the Na^+/H^+ exchanger plays a role in mediating GnRH-stimulated GTH-II release. One possible pathway in which GnRH may activate the Na^+/H^+ exchanger is through the activation of PKC. PKC has been demonstrated to be involved in both sGnRH- and cGnRH-II-stimulated GTH-II release (Chang *et al.*, 1993, 1996). In addition, PKC has been linked to Na^+/H^+ exchanger activity in some cell types (Noël and Pouysségur, 1995), including mammalian gonadotrophs (Bouali-Benazzouz, 1993). Therefore, to test the possible involvement of the Na^+/H^+ exchanger during PKC action, the GTH-II-release response to the PKC activator, TPA, in the presence or absence of amiloride or DMA was monitored under static-incubation conditions. Although 10 - 100 μM amiloride did not significantly alter 10 nM TPA-induced GTH-II release, the GTH-II response to 10 nM TPA was significantly reduced by 100 μM DMA (Fig. 4.6). In addition, both 0.1 and 1.0 nM TPA-stimulated GTH-II release were reduced by 100 μM DMA. Net GTH-II response to 0.1 nM TPA in the absence or presence of 100 μM DMA was 17.9 ± 6.5 and 0.0 ± 4.9 %, respectively; net GTH-II response to 1.0 nM TPA in the absence or presence of 100 μM DMA was 45.6 ± 8.0 and 24.1 ± 6.3 %, respectively ($P < 0.05$, unpaired t test, $n = 10$). Taken together, these results suggest that Na^+/H^+ exchange is involved in mediating sGnRH-, cGnRH-II- and PKC-stimulated GTH-II release.

Selectivity of amiloride and DMA action

It has been reported that amiloride and DMA reduce Ca^{2+} currents through voltage-sensitive Ca^{2+} channels in some cell types (Garcia *et al.*, 1990). Therefore, to determine if amiloride or DMA act on other signaling processes which may affect GTH-II release in the goldfish, I tested their effects on Ca^{2+}-induced GTH-II release, as well as voltage-dependent Ca^{2+} and Na^+ channel activity. The Ca^{2+} ionophore, ionomycin (10 μM), significantly stimulated GTH-II release in 2-hr static-incubation experiments (Fig. 4.7; $n = 16$). However, neither amiloride nor DMA altered ionomycin-stimulated GTH-II release, suggesting that they do not affect Ca^{2+}-dependent exocytotic events. As dihydropyridine-sensitive Ca^{2+} channels have been demonstrated to be involved in GnRH-stimulated GTH-II release, we also tested the possible effects of amiloride and DMA on dihydropyridine-sensitive Ca^{2+} channels in both hormone release and electrophysiological experiments. Incubation of dispersed pituitary cells with Bay K 8644 (10 μM), a dihydropyridine Ca^{2+} channel activator, significantly elevated GTH-II. In this experiment, 10 μM DMA elevated basal GTH-II levels. However, neither 10 nor 100 μM DMA altered the GTH-II response to Bay K 8644 (Fig. 4.8; net increase in GTH-II levels above basal in response to Bay K 8644: control, 70 ± 5%; 10 μM DMA, 57 ± 10%; 100 μM DMA, 66 ± 4%; $P > 0.05$ *vs.* each other, ANOVA; $n = 12$). Moreover, neither 100 μM amiloride nor 100 μM DMA altered the amplitude of Ba^{2+} currents recorded in identified gonadotrophs (Table 4.3 and Fig. 4.9A; $n = 5$). Similarly, the peak Na^+ current amplitude was not affected by either 100 μM amiloride or 100 μM DMA (Table 4.3 and Fig. 4.9B; $n = 9$). These results indicate that amiloride and DMA do not act on Ca^{2+}-dependent exocytotic events or voltage-dependent Ca^{2+} or Na^+ channels to inhibit GnRH-stimulated GTH-II release.

Discussion

The role of extracellular Ca^{2+} influx in regulating hormone release from the pituitary has been well established in many vertebrate species (Stojilkovic and Catt, 1992; Chang and Jobin, 1994a). In contrast, the function of extracellular Na^+ during pituitary hormone release has received much less attention, despite its involvement in cellular homeostasis (Grinstein and Rothsten, 1986), excitability (Hodgkin and Huxley, 1952), and modulation of exocytosis (Nordmann *et al.*, 1992). The role of extracellular Na^+ during pituitary LH release in mammals is still unclear. In ovine gonadotrophs, removal of extracellular Na^+ did not affect GnRH-stimulated LH release (Mason and Sikdar, 1988). In contrast, removal of extracellular Na^+ reduced GnRH-stimulated LH release from dispersed female rat pituitary cells; however, this effect was only observed in bicarbonate-free medium and therefore may not occur *in vivo* (McArdle *et al.*, 1991). In the present study on goldfish pituitary cells, GnRH-stimulated GTH-II release was attenuated in Na^+-depleted, bicarbonate-containing medium. It is unlikely that this reduction in GTH-II release results from an inhibition in GnRH-receptor binding, as preliminary results indicate that GnRH-receptor binding is normal in Na^+-free conditions (H. Habibi, personal communication). On the other hand, the reduction in GnRH-stimulated GTH-II release following the removal of extracellular Na^+ may indicate the involvement of voltage-sensitive Na^+ channels and/or Na^+/H^+ exchangers during GnRH action in goldfish. These possibilities were further investigated in electrophysiological and hormone release experiments.

Although the presence of TTX-sensitive Na^+ channels has been confirmed in all anterior pituitary cells studied (Stojilkovic and Catt, 1992), their relative role in regulating hormone release varies depending on the cell type, sex and species examined (Childs *et al.*, 1987; Cobbett *et al.*, 1987; Chen *et al.*, 1990; Chang and Jobin, 1994a). With respect to GnRH regulation of gonadotropin release, the action of TTX on agonist-induced LH release is still unclear. GnRH-stimulated LH release from the ovine (Mason and Sikdar, 1988) and frog (Porter and Licht, 1986) pituitary is unaffected by TTX, whereas TTX has been found to have either no effect (Conn and Rogers, 1980) or to augment GnRH-induced LH release from rat gonadotrophs (Waring and Turgeon, 1991). On the other hand, TTX has been reported to inhibit GnRH action on bovine gonadotrophs (Kile and Amoss, 1988). In addition, GnRH-induced action potentials in male rat gonadotrophs have been reported to be reduced by TTX (Tse and Hille, 1993). In the present study, GnRH-stimulated GTH-II release was unaffected by TTX and GnRH had no effect on the voltage-dependent properties of the Na^+ current. Therefore, there is no evidence to suggest that TTX-sensitive, voltage-dependent Na^+ channels play a role during GnRH-stimulated GTH-II release from goldfish gonadotrophs.

Although the effects of removal of extracellular Na^+ on GTH-II responses to the two GnRHs cannot be explained by the involvement of TTX-sensitive Na^+ channels, other mechanisms of Na^+ entry, such as the amiloride-sensitive Na^+/H^+ exchange system, may be affected. The Na^+/H^+ exchanger has been demonstrated to participate in the cellular responses to various hormones, particularly those which involve a rise in $[Ca^{2+}]_i$ (Grinstein and Rothsten, 1986; Noël and Pouysségur, 1995). For example, in rat adrenal glomerulosa cells (Conlin *et al.*, 1993) and gonadotrophs from female rats (Bouali-Benazzouz *et al.*, 1993), activation of the Na^+/H^+ exchanger is important in counteracting the cytosolic acidification produced by enhanced Ca^{2+} entry which occurs during agonist-induced hormone release. In addition, activation of PKC has been demonstrated to play a role in the regulation of the Na^+/H^+ exchanger activity in rat adrenal glomerulosa (Conlin *et al.*, 1993), gonadotropin (Bouali-Benazzouz *et al.*, 1993) and pineal (Ho *et al.*, 1989) cells. In goldfish gonadotrophs, both sGnRH and cGnRH-II stimulation of GTH-II release involves the activation of PKC and entry of Ca^{2+} through voltage-dependent Ca^{2+} channels (Chang *et al.*, 1993, 1996). The resulting increase in $[Ca^{2+}]_i$ would likely lead to acidification of the cytosol, as demonstrated in other cell types (Grinstein and Rothsten, 1986; Bouali-Benazzouz *et al.*, 1993; Noël and Pouysségur, 1995). Therefore, activation of the Na^+/H^+ exchanger, possibly by PKC, may be important in maintaining intracellular pH during GnRH action in goldfish gonadotrophs (Fig. 4.10). Although our results from experiments with inhibitors of Na^+/H^+ exchangers suggest that this antiport participates in the GTH-II-release response to both GnRHs and TPA, it is not yet known if alterations in cytosolic pH occur during these actions. In addition, other regulators of cellular pH, such as Na^+-dependent and/or Na^+-independent Cl^-/HCO_3^- exchangers may also be involved. As in other studies (McArdle *et al.*, 1991; Bouali-Benazzouz *et al.*, 1993), Na^+/H^+ exchangers do not appear to be involved during basal GTH-II release from goldfish gonadotrophs.

Both male and female goldfish from all stages of the seasonal gonadal cycle were used in the present study. Previous studies have demonstrated that the greatest increases in serum GTH-II to GnRH were found in prespawning goldfish, while fish that were sexually regressed were the least responsive (Habibi *et al.*, 1989). In addition, basal GTH-II release and the number of high affinity GnRH receptors has been demonstrated to vary seasonally (Peter *et al.*, 1991). Irrespective of these possible qualitative and quantitative changes in gonadotroph cell function, GnRH-stimulated GTH-II release was reduced in the presence of Na^+-depleted medium, amiloride or DMA during all stages of the gonadal reproductive cycle. This suggests that the extracellular Na^+-dependence and the involvement of Na^+/H^+ exchangers in GnRH action are important events at all stages of the reproductive cycle in goldfish. However, whether or not gonadal steroids and gonadal status alter the activity of the Na^+/H^+ exchanger in goldfish gonadotrophs, as they do in female rat gonadotrophs (Bouali-Benazzouz *et al.*, 1993), requires further study.

Amiloride and DMA at the concentrations used are known to be effective inhibitors of the Na^+/H^+ exchanger (Hamill *et al.*, 1992). However, it has been reported that they also can block "L-type" Ca^{2+} channels in some (Garcia *et al.*, 1990), but not in other studies (Thompson and Wong, 1991; Allan *et al.*, 1993). As "L-type" Ca^{2+} channels and increases in $[Ca^{2+}]_i$ play a pivotal role in GnRH-stimulated GTH-II release from goldfish gonadotrophs, we examined the actions of amiloride and DMA on voltage-sensitive Ca^{2+} currents and on Ca^{2+}-stimulated exocytosis. Neither amiloride nor DMA had any effect on voltage-sensitive Ca^{2+} currents. Moreover, in static-incubation experiments, DMA did not affect the ability of the "L-type" Ca^{2+} channel agonist, Bay K 8644, to stimulate GTH-II release. Similarly, neither amiloride nor DMA affected the GTH-II release response to the Ca^{2+} ionophore, ionomycin. In addition to their inability to alter Ca^{2+} currents or Ca^{2+}-dependent exocytotic events, neither amiloride nor DMA affected the voltage-dependent Na^+ currents in goldfish gonadotrophs. These results strongly indicate that the inhibitory action of amiloride and DMA on GnRH-stimulated GTH-II release is not mediated through an inhibition of "L-type" Ca^{2+} channels, voltage-dependent Na^+ channels, or Ca^{2+}-dependent exocytosis.

Finally, to investigate the role of Na^+ influx on GTH-II release, we used the Na^+ channel modulator veratridine, which allows Na^+ to move passively through voltage-dependent Na^+ channels, resulting in increased cytosolic Na^+ concentrations and membrane depolarization (Catterall and Nirenberg, 1973). Addition of veratridine in Na^+- and Ca^{2+}-containing medium resulted in an increase in GTH-II release. Under these conditions, the resulting GTH-II release is likely due in part to the activation of "L-type" Ca^{2+} channels caused by the veratridine-induced membrane depolarization. As expected, removal of extracellular Ca^{2+} resulted in a decrease in the GTH-II response to veratridine. These results are consistent with the view that Ca^{2+} influx through voltage-sensitive Ca^{2+} channels is involved in GTH-II release from the goldfish pituitary (Jobin and Chang, 1992a). However, veratridine still retained approximately 40% of its GTH-II-releasing activity under extracellular Ca^{2+} free conditions. These data indicate that Na^+ entry, independent of extracellular Ca^{2+} entry, can increase GTH-II release. Several explanations could account for this result: 1. Na^+- or depolarization-induced Ca^{2+} release from intracellular stores (Nordmann *et al.*, 1992; Berridge, 1993; Zucker, 1993); 2. direct effects of membrane depolarization on exocytosis in the absence of extracellular Ca^{2+} influx (Hochner *et al.*, 1989); 3. altered Na^+/Ca^{2+} exchanger activity (Zucker, 1993); and iv. a direct effect of Na^+ on release (Nordmann *et al.*, 1992). It remains to be determined which of these factors are involved in veratridine-induced GTH-II release from goldfish gonadotrophs.

In summary, extracellular Na^+ is involved in GnRH-evoked GTH-II release. The extracellular Na^+-dependence in GnRH-stimulated GTH-II release does not involve voltage-sensitive Na^+ channel activity, but is partially a result of the participation of amiloride-sensitive Na^+/H^+ exchangers. Activation of Na^+/H^+ exchanger activity by GnRH is likely mediated by PKC. Unlike the situation in mammalian gonadotrophs (McArdle *et al.*, 1991), the effects of Na^+-depleted media, amiloride and DMA on GTH-II release were observed in bicarbonate-containing media. Thus, it is likely that activity of a Na^+/H^+ exchanger is an important component in GnRH action in goldfish, even in conditions where the pH buffering capacity has not been compromised. To our knowledge, these studies are the first to implicate the involvement of extracellular Na^+ and Na^+/H^+ exchangers during GnRH-stimulated GTH-II release in lower vertebrates. In addition, this is the first study to indicate that Na^+ influx may stimulate the release of gonadotropin in the absence of extracellular Ca^{2+}.

Table 4.1. Extracellular Na^+ dependence of PKC-stimulated GTH-II release from dispersed goldfish pituitary cells in static culture.

TPA (nM)	Net GTH-II response compared to unstimulated controls (%)[a]	
	Normal medium	Na^+-depleted medium
0	0.0 ±1.6[b]	0.0 ±3.0[b]
0.1	8.5 ± 3.4[b]	10.5 ± 3.9[b]
1.0	143.6 ± 8.4	98.9 ± 9.9[c]
10.0	308.4 ± 13.6	257.9 ± 17.3[c]

[a] Average basal values = 99.1 ± 7.2 ng/ml/.25 X 10^6 cells. All experiments were performed with fish at gonadal recrudescence.

[b] Within the same medium group, GTH-II values (mean ± SEM, $n = 16$) which are similar ($P > 0.05$; ANOVA, followed by LSD test) are identified by the same superscript.

[c] Significantly different from responses in normal medium ($P < 0.05$; unpaired t-test).

Table 4.2. GnRH effects on the steady-state inactivation properties of the Na^+ current.

A.

Inactivation properties (equation 1)	Control	100 nM sGnRH *n* = 4	Washout
$E_{1/2}$ (mV)	-63.8 ± 1.3	-63.1 ± 0.6	-64.3 ± 0.4
k	8.1 ± 0.5	7.0 ± 0.2	7.5 ± 0.2

B.

Inactivation properties (equation 1)	Control	100 nM cGnRH-II *n* = 3	Washout
$E_{1/2}$ (mV)	-60.4 ± 1.0	-62.4 ± 1.6	-64.2 ± 1.0
k	7.5 ± 0.4	7.9 ± 0.6	8.2 ± 0.4

All experiments were performed with gonadotrophs prepared from sexually regressed and recrudescent fish.

Table 4.3. Amiloride (A) and DMA (B) effects on inward Na^+ and Ba^{2+} currents elicited by a 40 msec voltage step to -10 mV (for Na^+ current) or 0 mV (for Ba^{2+} current) from a holding potential of -80 mV.

A.

Current (pA)	Control	100 µM Amiloride	Washout
Na^+ ($n = 9$)	-325.7 ± 49.2	-331.1 ± 48.9	-338.9 ± 50.9
Ba^{2+} ($n = 5$)	-39.4 ± 6.1	-34.2 ± 4.7	-32.5 ± 4.5

B.

Current (pA)	Control	100 µM DMA	Washout
Na^+ ($n = 9$)	-319.7 ± 44.3	-310.1 ± 42.7	-308.2 ± 59.2
Ba^{2+} ($n = 5$)	-45.1 ± 5.8	-43.4 ± 5.5	-41.3 ± 6.1

All experiments were performed with gonadotrophs prepared from prespawning and regressed fish.

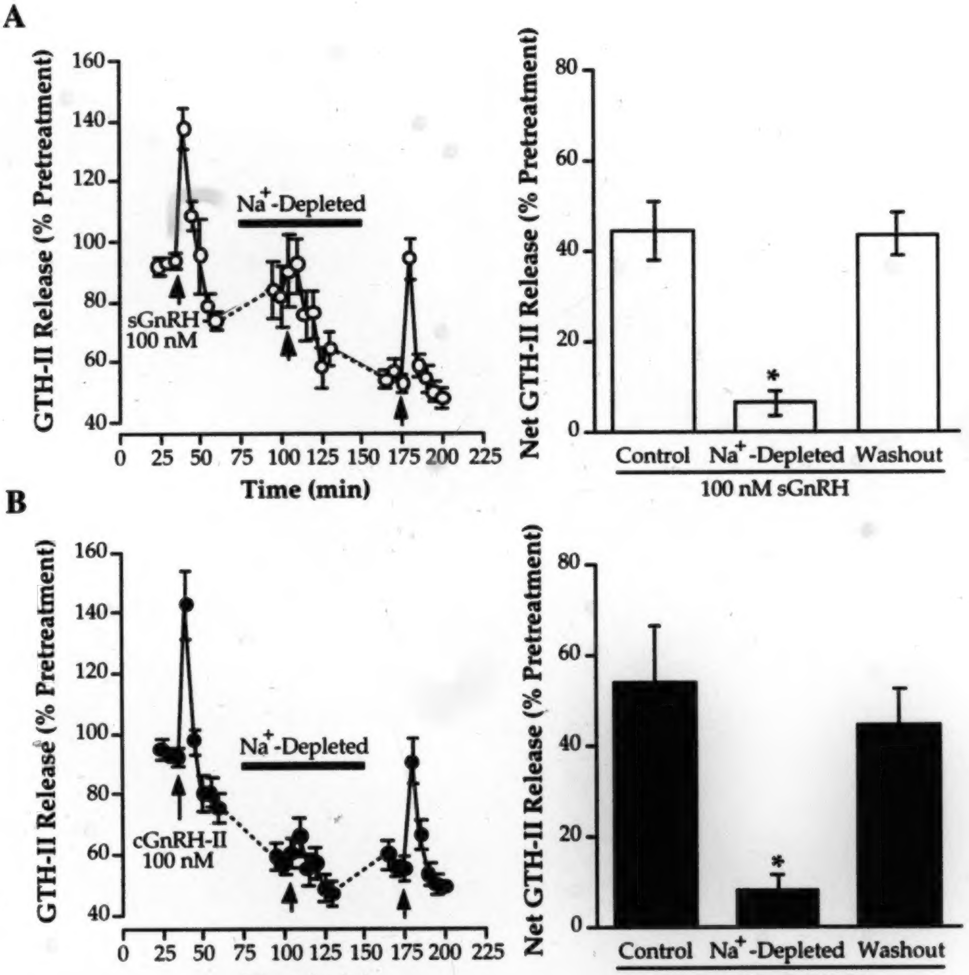
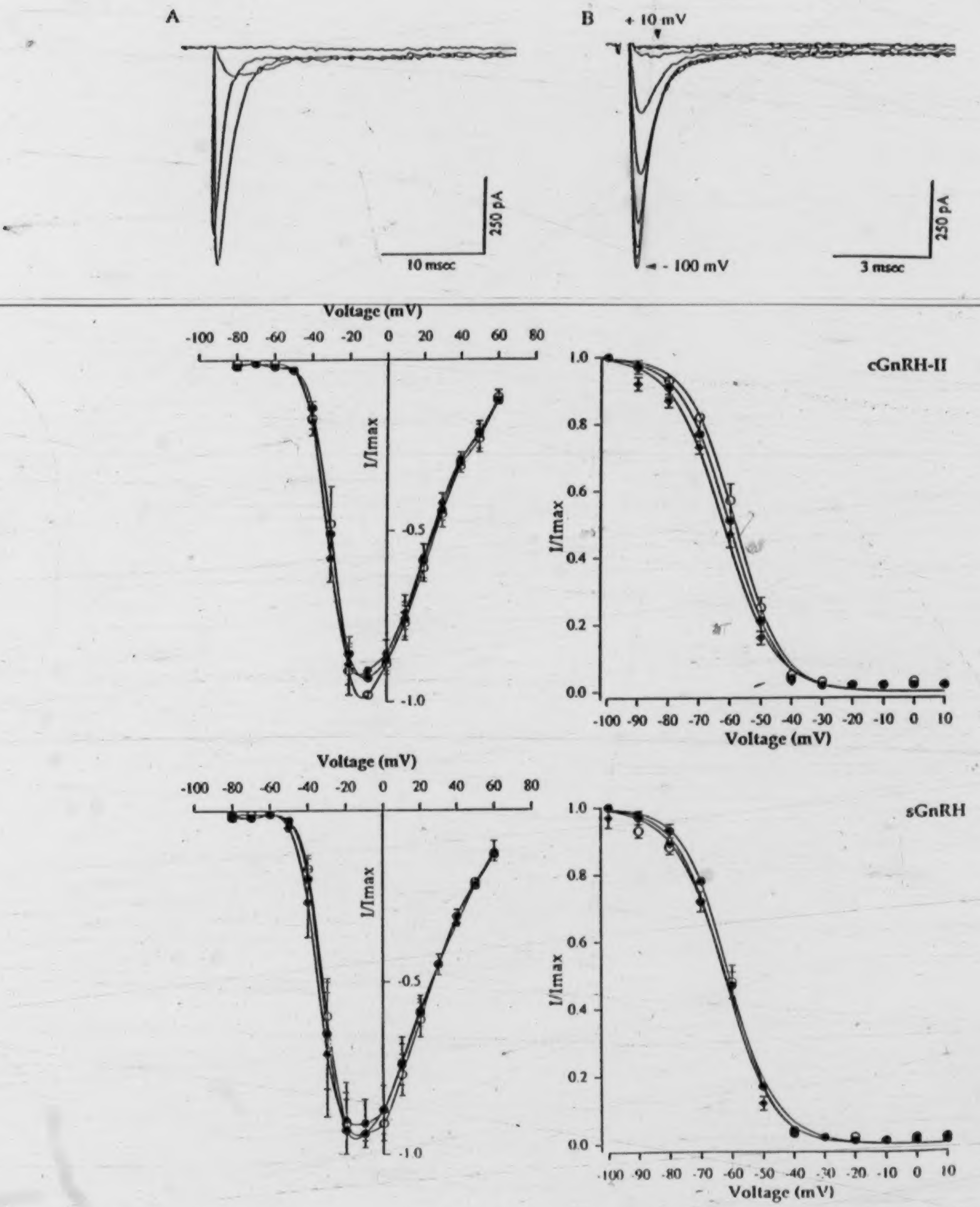


Fig. 4.1. Extracellular Na$^+$ dependence of GnRH-stimulated GTH-II release from perifused goldfish pituitary cells. A. Left panel: hormone-release response to a 5 min pulse of 100 nM sGnRH (*arrow*) in normal and Na$^+$-depleted conditions (*bar*; $n = 12$). Right panel: net GTH-II response to 100 nM sGnRH before (control), during (Na$^+$-depleted) and after (washout) perifusion with Na$^+$-depleted media. B. Left panel: hormone-release response to a 5 min pulse of 100 nM cGnRH-II (*arrow*) in normal and Na$^+$-depleted conditions (*bar*; $n = 8$). Right panel: net GTH-II release response to 100 nM cGnRH-II before (control), during (Na$^+$-depleted) and after (washout) perifusion with Na$^+$-depleted media. Results from experiments using pituitary cells prepared from sexually regressed, recrudescent and prespawning fish were normalized as a percentage of pretreatment values (17.82 ± 0.08 ng/ml/2 x 10^6 cells) and presented as mean ± SEM. *Asterisks* denote significant differences from the control and washout responses to GnRH ($P < 0.05$, by ANOVA and Fisher's LSD test).

Fig. 4.2. Effects of GnRH on the voltage-dependent Na$^+$ current in identified goldfish gonadotrophs. A. The current-voltage relationship of the Na$^+$ current was obtained by holding the cells at -80 mV and then stepping for 50 msec to command potentials between -80 and +80 mV, in 10 mV increments. Upper panel: representative current traces elicited during command potentials to -80, -30, -10 and +10 mV. Middle panel: current-voltage relationship of the Na$^+$ current before (*open circles*), during (*filled circles*) and after (*filled diamonds*) application of 100 nM sGnRH (n = 3). Lower panel: current-voltage relationship of the Na$^+$ current before (*open circles*), during (*filled circles*) and after (*filled diamonds*) application of 100 nM cGnRH-II (n = 4). All currents were normalized to the maximal inward current before the application of GnRH and are presented as mean ± SEM. B. Steady-state inactivation curves for the voltage-dependent Na$^+$ current were generated by stepping the membrane potential to between -100 and +10 mV for 100 msec prior to stepping to a 20 msec test potential of +0 mV. Upper panel: representative current traces of the remaining current elicited by a test pulse to +8 mV following prepulse potentials between -100 and +10 mV. Middle panel: inactivation curve for the voltage-dependent Na$^+$ current before (*open circles*), during (*filled circles*) and after (*filled diamonds*) application of 100 nM sGnRH (n = 3). Lower panel: inactivation curve for the voltage-dependent Na$^+$ current before (*open circles*), during (*filled circles*) and after (*filled diamonds*) application of 100 nM cGnRH-II (n = 4). The solid line for each of the steady-state inactivation curves is a fitted Boltzman relation (equation 1). All currents were normalized to the maximal inward current and are presented as mean ± SEM. All current recordings were carried out in identified gonadotrophs prepared from sexually regressed and recrudescent fish.


A
B
+ 10 mV
- 100 mV
250 pA
10 msec
250 pA
3 msec
Voltage (mV)
-100 -80 -60 -40 -20 0 20 40 60 80
I/Imax
-0.5
-1.0
1.0
0.8
0.6
0.4
0.2
0.0
I/Imax
cGnRH-II
-100 -90 -80 -70 -60 -50 -40 -30 -20 -10 0 10
Voltage (mV)
Voltage (mV)
-100 -80 -60 -40 -20 0 20 40 60 80
I/Imax
-0.5
-1.0
1.0
0.8
0.6
0.4
0.2
0.0
I/Imax
sGnRH
-100 -90 -80 -70 -60 -50 -40 -30 -20 -10 0 10
Voltage (mV)

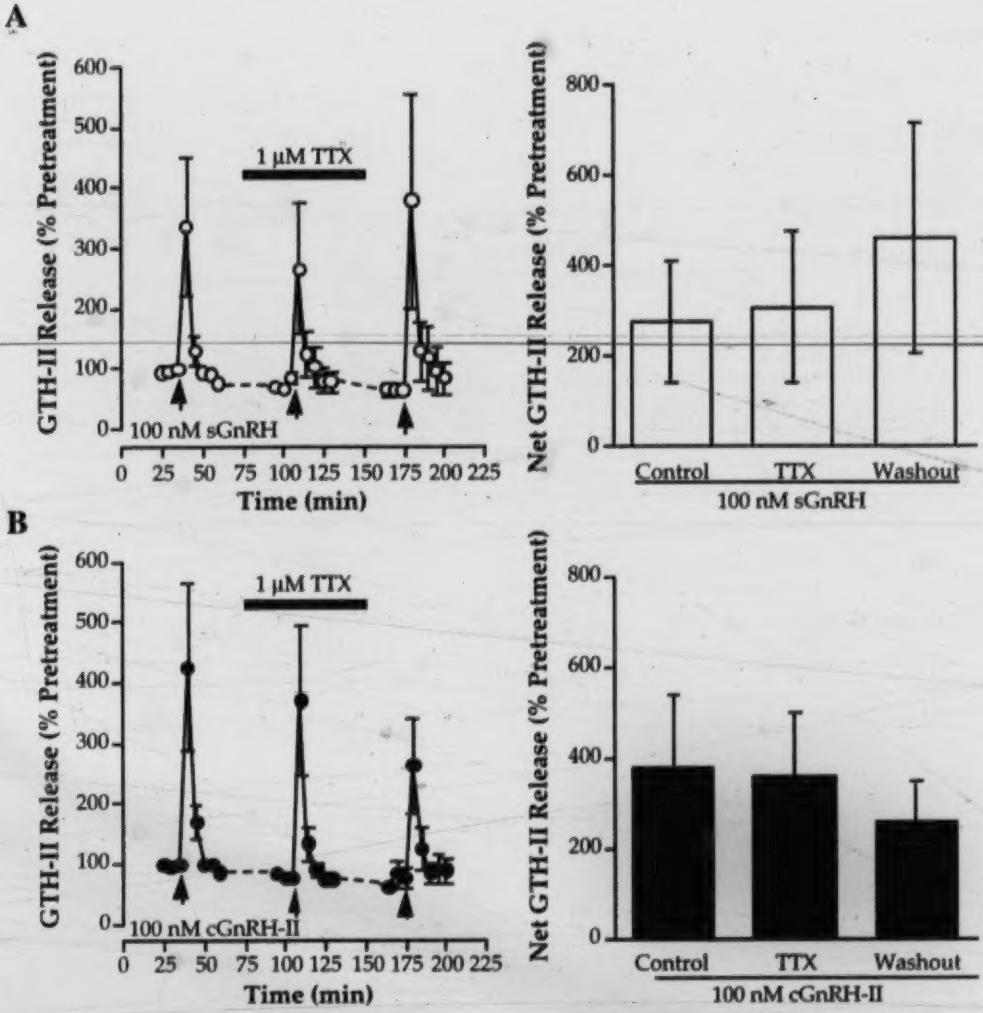


Fig. 4.3. Lack of TTX-sensitivity during GnRH-stimulated GTH-II release from perifused goldfish pituitary cells. A. Left panel: hormone-release response to a 5 min pulse of 100 nM sGnRH (*arrow*) in normal and 1 µM TTX-containing medium (*bar*; *n* = 4). Right panel: net GTH-II response to 100 nM sGnRH before (control), during (TTX) and after (washout) perifusion with 1 µM TTX. B. Left panel: hormone-release response to a 5 min pulse of 100 nM cGnRH-II (*arrow*) in normal and 1 µM TTX-containing medium (*bar*; *n* = 7). Right panel: net GTH-II response to 100 nM cGnRH-II before (control), during (TTX) and after (washout) perifusion with 1 µM TTX. Results from experiments using pituitary cells prepared from sexually recrudescent and prespawning fish were normalized as a percentage of pretreatment values (16.12 ± 0.51 ng/ml/2 x 10^6 cells) and presented as mean ± SEM.

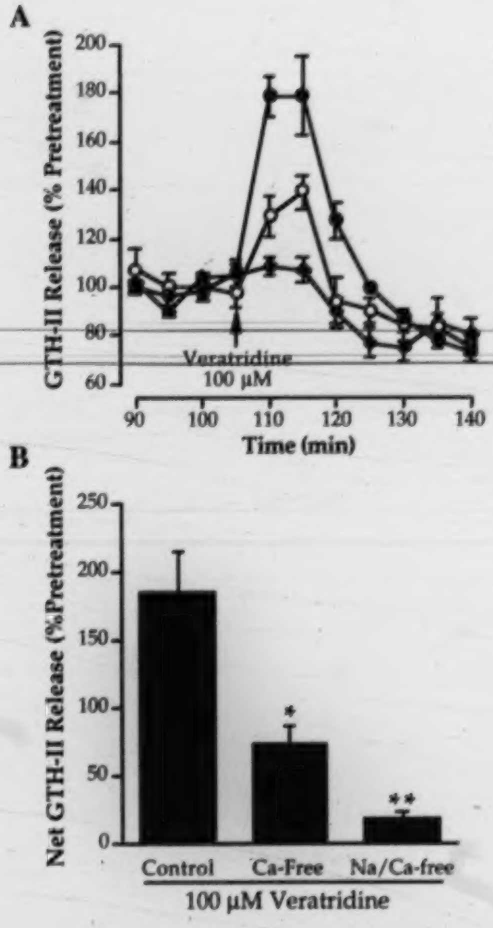


Fig. 4.4. Veratridine-stimulated GTH-II release from perifused goldfish pituitary cells. A. Hormone release response to a 5 min pulse of 100 µM veratridine (*arrow*) in normal (*filled circles*), Ca^{2+}-free medium (*open circles*) and Na^{+}-depleted/Ca^{2+} free medium (*filled diamonds; n* = 8). B. Net GTH-II response to 100 µM veratridine in normal (control), Ca^{2+}-free medium (Ca-Free) and Na^{+}-depleted/Ca^{2+} free medium (Na/Ca-free). Results from experiments using pituitary cells prepared from sexually regressed, recrudescent and prespawning fish were normalized as a percentage of pretreatment values (20.17 ± 0.38 ng/ml/2 x 10^6 cells) and presented as mean ± SEM. *Asterisks* denotes significant differences from control; double *asterisks* denotes sigificant difference from control and Ca^{2+} free ($P < 0.05$, by ANOVA and Fisher's LSD test). Treatment with Ca-free and Na^{+}-depleted/Ca^{2+}-free medium began at time = 90 min and continued for 55 min.

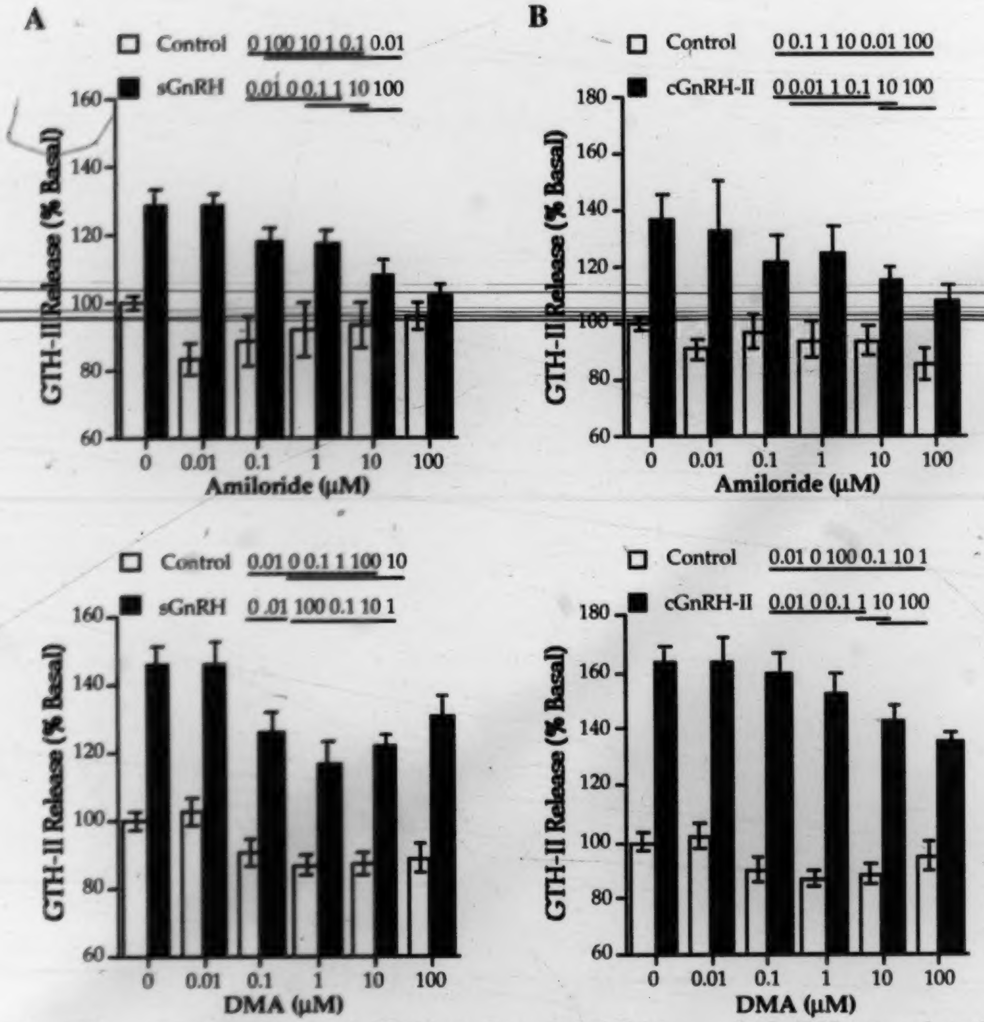


Fig. 4.5. Amiloride- and DMA-induced inhibition of GnRH-stimulated GTH-II release from dispersed pituitary cells in static culture. A. Effects of amiloride (upper panel: *n* = 24) and DMA (lower panel: *n* = 16) on 100 nM sGnRH-stimulated GTH-II release. B. Effects of amiloride (upper panel: *n* = 12) and DMA (lower panel: *n* = 20) on 100 nM cGnRH-II-stimulated GTH-II release. Results from experiments using pituitary cells prepared from sexually regressed, recrudescent and prespawning fish were normalized as a percentage of basal GTH-II release (255.2 ± 13.6 ng/ml/0.25 x 10^6 cells) and are presented as mean ± SEM. Treatment groups having similar GTH-II responses (ANOVA and Fisher's LSD, $P > 0.05$) are identified by the same underscore.

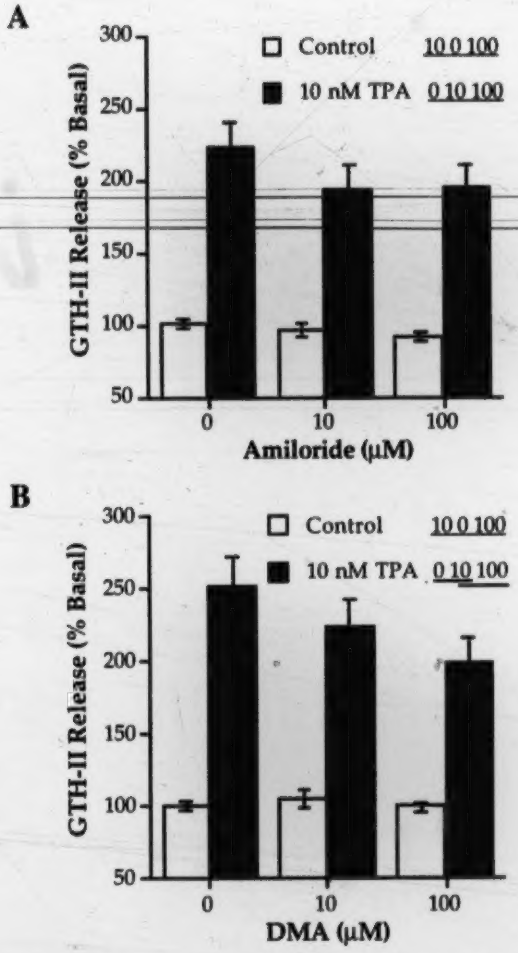


Fig. 4.6. Effects of amiloride (A) and DMA (B) on 10 nM TPA-stimulated GTH-II release from dispersed pituitary cells in static culture. Results from experiments using pituitary cells prepared from prespawning and regressed goldfish were normalized as a percentage of basal GTH-II release (178.0 ± 11.6 ng/ml/0.25 x 10^6 cells) and presented as mean ± SEM ($n = 12$ for each group). Treatment groups having similar GTH-II responses (ANOVA and Fisher's LSD, $P > 0.05$) are identified by the same underscore.

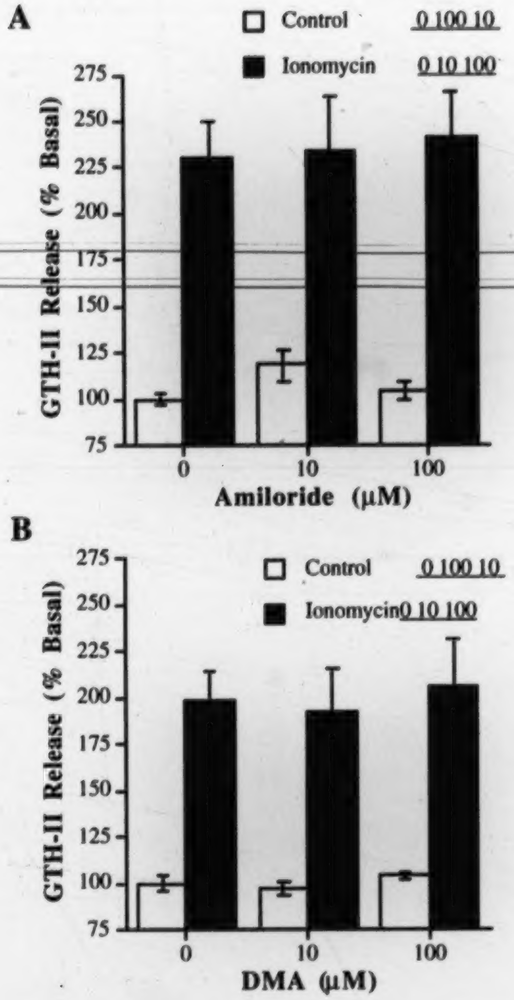


Fig. 4.7. Lack of effect of amiloride (A) and DMA (B) on 10 μM ionomycin-stimulated GTH-II release from dispersed pituitary cells in static-culture. Results from experiments using pituitary cells prepared from prespawning and regressed goldfish were normalized as a percentage of basal GTH-II release (420.0 ± 30.0 ng/ml/0.25 x 10^6 cells) and presented as mean ± SEM (n = 12). Treatment groups having similar GTH-II responses (ANOVA and Fisher's LSD, $P > 0.05$) are identified by the same underscore.

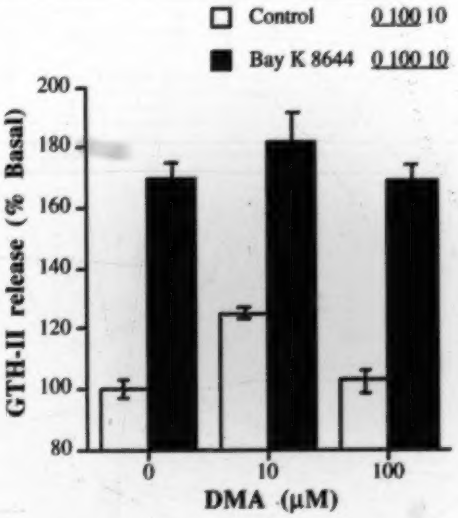


Fig. 4.8. Lack of effect of DMA on 10 μM Bay K 8644-stimulated GTH-II release from dispersed pituitary cells in static-culture (*n* = 12). Results from experiments using pituitary cells prepared from prespawning and regressed goldfish were normalized as a percentage of basal GTH-II release (165.4 ± 10.5 ng/ml/0.25 x 10^6 cells) and are presented as mean ± SEM. Treatment groups having similar GTH-II responses (ANOVA and Fisher's LSD, $P > 0.05$) are identified by the same underscore.

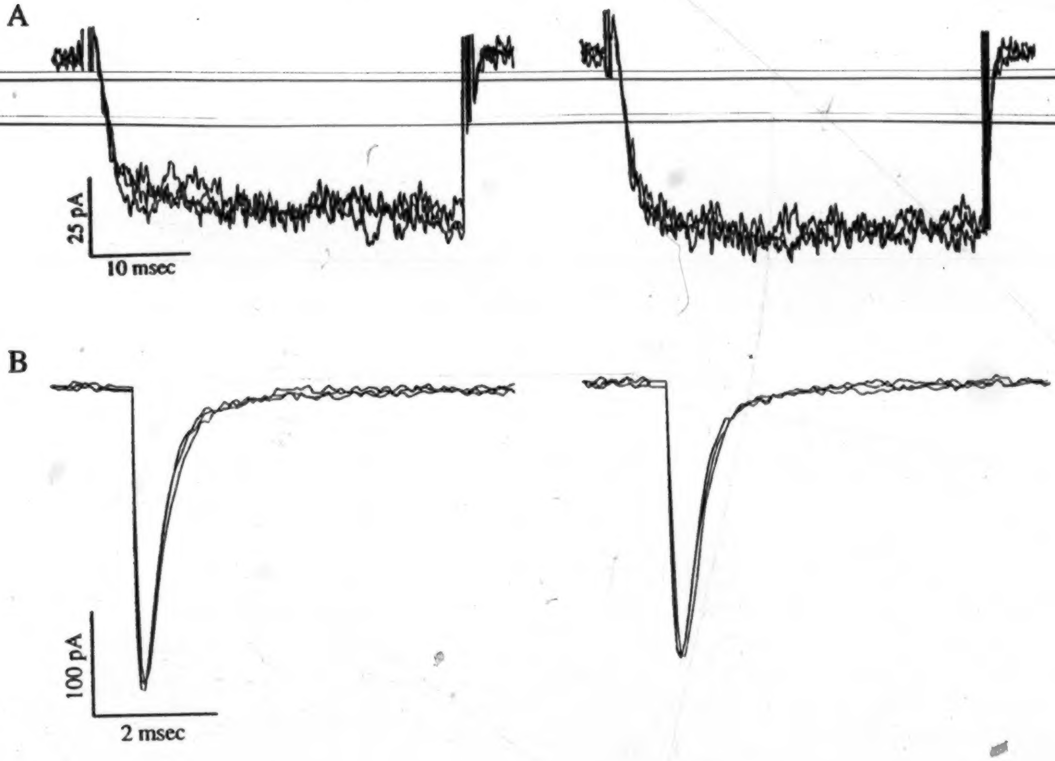


Fig. 4.9. Effects of amiloride or DMA on voltage-dependent Na^+ and Ba^{2+} currents in identified goldfish gonadotrophs. A. Superimposed Ba^{2+} current traces elicited during a 40 msec voltage step to +0 mV (holding potential = - 80 mV) before, during and after the application of 100 µM amiloride (left panel) or 100 µM DMA (right panel). B. Superimposed Na^+ current traces elicited during a 40 msec voltage step to - 10 mV (holding potential = - 70 mV) before, during and after the application of 100 µM amiloride (left panel) or 100 µM DMA (right panel). All current recordings were carried out using identified gonadotrophs prepared from prespawning and regressed goldfish.

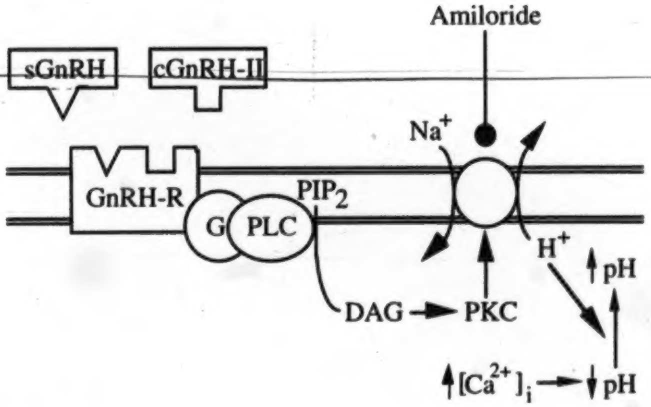


Fig. 4.10. Proposed model for pH regulation during GnRH-receptor activation in goldfish gonadotrophs. An increase or decrease in [Ca^{2+}]$_i$ or pH is indicated by upwards or downward facing arrows, respectively. A stimulatory or inhibitory response is indicated by a + or - sign, respectively.

Chapter 5

DOPAMINE-D2 ACTIONS ON VOLTAGE-DEPENDENT IONIC CURRENTS IN IDENTIFIED GOLDFISH GONADOTROPHS

INTRODUCTION

In goldfish, secretion of gonadotropin-II (GTH-II), the functional equivalent of luteinizing hormone, is under stimulatory and inhibitory control by GnRH and dopamine, respectively (for review, see Peter *et al.*, 1986; Peter *et al.*, 1991; Chang *et al.*, 1993). GnRH is delivered directly to gonadotrophs in the proximal pars distalis by hypothalamic innervation of neurosecretory fibers (Peter *et al.*, 1990b; Kah *et al.*, 1993). The GnRH nerve terminals are close to gonadotrophs (Kah *et al.* 1993), where they release two endogenous GnRH forms, sGnRH and cGnRH-II. Both GnRHs, bind to the same class of high affinity, low capacity receptors to stimulate GTH-II secretion (Peter *et al.*, 1991; Chang *et al.*, 1996). Hypothalamic dopaminergic neurons also innervate the proximal pars distalis (Peter *et al.*, 1991). Dopamine release from these nerve terminals directly inhibits GnRH-stimulated GTH-II secretion by the activation of dopamine D_2-like receptors (Peter *et al.*, 1986; Chang *et al.*, 1993). The direct regulation of GTH-II secretion in goldfish by GnRH and dopamine provides an excellent model system to study interactions at the pituitary level between stimulatory and inhibitory neuroendocrine factors.

Whereas the intracellular signaling mechanisms mediating GnRH action in goldfish are partially understood (reviewed in Chang *et al.*, 1993; Chang and Jobin 1994a; Chang *et al.*, 1996), less is known about the mechanism of dopmine action. The two native GnRH forms stimulate GTH-II secretion via a PKC/Ca^{2+}-dependent signaling pathway (Chang and Jobin, 1994a; Jobin *et al.*, 1996; Chang *et al.*, 1996). Salmon GnRH and cGnRH-II activate PKC, which in turn increases Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channels (Jobin and Chang, 1992a, 1992b). The resulting elevation in cytosolic free Ca^{2+} concentration $[Ca^{2+}]_i$ is believed to play a central role in GTH-II secretion. In contrast to GnRH, dopamine acts directly at the level of the pituitary to inhibit GTH-II secretion through undefined mechanisms (Peter *et al.*, 1986; Peter *et al.*, 1991; Chang *et al.*, 1993). Given the importance of extracellular Ca^{2+} influx in the mediation of GnRH-induced GTH-II secretion, a distinct possibility is that dopamine reduces voltage-dependent Ca^{2+} channel activity to inhibit GTH-II secretion. In preliminary studies using mixed populations of dispersed goldfish pituitary cells, dopamine agonists reduced GnRH-stimulated increases in $[Ca^{2+}]_i$ (Chang *et al.*, 1993). There is also evidence from other pituitary cell models demonstrating that dopamine alters plasma membrane electrical activity to regulate pituitary cell function. In lactotrophs from the teleost fish alewife (*Alosa pseudoharengus*), dopamine inhibits Na^+/Ca^{2+}-dependent action potential activity (Taraskevich and Douglas, 1978). Additionally, in rat lactotrophs, rat melanotrophs and frog melanotrophs, dopamine modulates a variety of ionic channels to arrest plasma membrane electrical activity, resulting in a reduction in Ca^{2+} influx and hormone secretion (Malgaroli *et al.*, 1987; Stack and Surprenant, 1991; Keja *et al.*, 1992; Vaudry *et al.*, 1994).

In Chapter 3, tetrodotoxin-sensitive Na^+ currents, L-type Ca^{2+} currents, and multiple K^+ current subtypes were observed in identified goldfish gonadotrophs, and their voltage-dependent and pharmacological properties were characterized using whole-cell voltage-clamp recordings. To begin to test the hypothesis that dopamine inhibits GTH-II secretion by modulating voltage-dependent Ca^{2+} channels, the effects of dopamine on voltage-dependent ionic currents were characterized in identified goldfish gonadotrophs. The receptor sub-type mediating dopamine action on ionic currents was also examined using dopamine D_1-like and D_2-like receptor agonists and antagonists. Using both hormone release and electrophysiological techniques, it was demonstrated in the present study that dopamine D_2 receptor activation reduces Ca^{2+} influx through voltage-dependent Ca^{2+} channels, resulting in an inhibition of GTH-II secretion. Unlike voltage-dependent Ca^{2+} currents, voltage-dependent Na^+ and K^+ currents were not affected by dopamine or dopamine receptor agonists. It is proposed that inhibition of voltage-dependent Ca^{2+} channels mediates the inhibitory neuroendocrine action of dopamine on GTH-II secretion from goldfish gonadotrophs. Moreover, these studies identify an important signaling pathway whereby inhibitory neuroendocrine factors can regulate GTH secretion in general.

MATERIALS AND METHODS

Animals

Common goldfish (*Carassius auratus*; 8-13 cm in body length) were purchased from Grassyforks Fisheries (Martinsville, IN, USA) or Ozark Fisheries (Stoutland, MO, USA) and held in flow-through aquaria (1800 l).

Fish were maintained at 17-20 °C on a simulated natural photoperiod (adjusted weekly according to the times of sunset and sunrise in Edmonton, AB, Canada) and fed to satiation with commercial fish food daily. Fish were acclimated to the above conditions for at least seven days prior to use. Sexually regressed, recrudescent and mature (pre-spawning) male and female goldfish were used in the present study. The effects of dopamine and dopamine agonists were similar in replicate experiments performed at different times of the seasonal reproductive cycle and therefore, pooled results are presented.

Dispersion of pituitary cells

Fish were anesthetized in 0.05% tricaine methanesulfonate prior to decapitation. Pituitaries from both male and female goldfish were removed and placed in dispersion medium (Medium 199 with Hanks' salts, 25 mM HEPES, 2.2 g/l $NaHCO_3$, 0.3% BSA, 100 000 U/l penicillin, and 100 mg/l streptomycin, pH adjusted to 7.2 with NaOH; Gibco, Grand Island, NY, USA). Pituitary cells were dispersed using a controlled trypsin/DNAse treatment procedure modified from Chang *et al* (1990a). The specific modification was that 0.15% trypsin was used in the cell preparation instead of 0.25% trypsin.

Static culture

For measurements of prolonged hormone release responses to various stimulators and inhibitors, dispersed goldfish pituitary cells were resuspended in plating medium (Medium 199 with Earle's salts, 25 mM HEPES, 2.2 g/l $NaHCO_3$, 100 000 U/l penicillin and 100 mg/l streptomycin, pH adjusted to 7.2 with NaOH; Gibco), supplemented with 1 % horse serum. Static culture studies were performed as previously described (Chang *et al.*, 1990a). All treatments were tested in quadruplicate and each experiment was repeated a minimum of three times. Results from replicate experiments were expressed as a percentage of basal release (unstimulated control) and pooled data (mean ± SEM) are presented. Statistical analyses were performed using analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test or unpaired *t* test. Differences between groups were considered significant when $P < 0.05$.

Cell column perifusion studies of GTH-II responses

For the measurement of short-term hormone release responses to various stimulators and inhibitors, cell column perifusion studies were performed as previously described (Chapter 4; Chang *et al.*, 1990b). Experiments began with the collection of 5-min. fractions of perifusate for 40 min. At 40 min a 5-min pulse of 30 mM K^+ was given and the duration of each perifusate fraction was shortened to 30 sec to increase the resolution of the GTH-II response. Taking the dead volume and the flow rate into account, an immediate GTH-II response to K^+ stimulation would occur ≈ 7 min following the commencement of the K^+ pulse. Treatment with inhibitors commenced at 20 min and continued for a total duration of 40 min. Perifusates were kept frozen at -20 °C until their GTH-II contents could be measured using a radioimmunoassay specific for GTH-II (Peter *et al.*, 1984; Van Der Kraak *et al.*, 1992).

GTH-II content in individual columns was normalized as a percentage of the average pretreatment values obtained in the first four fractions collected (% Pretreatment). This data transformation was done to account for fluctuations in basal hormone release observed throughout the year and to allow pooling of the data from separate columns of the same experiment, without distorting the profile of hormone release during the course of perifusion. The hormone responses were quantified by determining the net increase in GTH-II levels (*i.e.* area under the curve) and expressed as a percentage of pretreatment values as previously described (Wong *et al.*, 1992). All values in the text and figures are reported as mean ± SEM. Differences between groups were considered to be significant when $P < 0.05$ using paired or unpaired t test.

For the present perifusion experiments, a modified testing medium was used (M-199 with Hank's salts prepared without the addition of NaCl or $CaCl_2$; courtesy of Dr. S.S. Stojikovic, ERRB, NICHD, National Institutes of Health, Bethesda, MD; Na^+, Ca^{2+} and K^+ levels were adjusted as required, see below). Under control conditions, the extracellular medium contained 136 mM NaCl, 5.4 mM KCl and 1.26 mM $CaCl_2$. For experiments using high extracellular K^+ concentrations, 25 mM NaCl was replaced with equimolar KCl, resulting in an extracellular KCl concentration of 30.4 mM. For experiments in which Ca^{2+}-free medium was required, $CaCl_2$-free medium with the addition of 0.1 mM EGTA was used. All solutions were supplemented with 2.2 g/l $NaHCO_3$, 25 mM HEPES, 100 000 U/l penicillin, 100 mg/l streptomycin, 0.1% BSA and pH was adjusted to 7.2 with TrisCl.

Electrophysiological Recordings

For electrophysiological studies, dispersed goldfish pituitary cells (0.25 x 10^6 cells/ml) were plated for 16 hr on poly-L-lysine-coated (0.01 mg/ml) glass-bottom petri dishes in plating medium supplemented with 10% horse serum and under the conditions described above. The use of 10% horse serum in overnight culture enhances cell responsiveness, but because of problems of cell detachment (Chang *et al.*, 1990a; Chang and Jobin, 1994b), this higher concentration of horse serum was not used for cell culture in hormone release experiments. Gonadotrophs and somatotrophs were identified on the basis of their unique morphological characteristics as previously described (Chapter 2). Additionally, co-localization of prolactin or GH does not occur in identified gonadotrophs (Chapter 2), indicating that the effects of dopamine on gonadotroph cell function observed in this study can only be attributed to the control of GTH-secretion.

Whole-cell, voltage-clamp recordings (Hamill *et al.*, 1981) were performed at room temperature (18 °C to 20 °C) using nystatin-perforated patch recording techniques (Korn and Horn, 1989) as described in chapter 3. Breifly, after obtaining electrical access to the cell ($R_S = 24.6 \pm 0.6$ MΩ; $n = 48$), currents were recorded in voltage-clamp mode using a Dagan 3900 integrating patch-clamp amplifier. Series resistance compensation was optimized and usually ranged between 10 and 18 mΩ. Current records were corrected for linear leakage and capacitance using a P/-4 procedure (Bezanilla and Armstrong, 1977). Pulse generation, and data acquisition were carried out using an AT 486 2DX compatible computer equipped with a digidata 1200 interface in conjunction with pCLAMP programs (Axon Instruments, Foster City, CA). The effects of all drug applications on ionic currents were continuously monitored by giving a 40 msec voltage-step to +10 mV from a holding potential of -80 mV, every 10 sec. Current-voltage relationships and steady-state inactivation curves were generated only after the effect of the drug application had stabilized.

The composition (in mM) of the external solutions for the different recording conditions are listed as follows:

1. Na^+ currents: 136 NaCl, 1.26 $CaCl_2$, 2.5 KCl, 1 $MgCl_2$, 0.05 $CdCl_2$, 10 tetraethylammonium (TEA)-Cl, 8 glucose and 10 HEPES (pH adjusted to 7.2 with NaOH).

2. Ba^{2+} currents (through voltage-dependent Ca^{2+} channels): 130 N-methyl-D-glucamine-Cl, 20 $BaCl_2$, 1.0 $MgCl_2$, 2.5 KCl, 8 glucose, 10 HEPES (pH adjusted to 7.2 with HCl).

3. K^+ currents: 142 NaCl, 6.0 $CaCl_2$, 2.0 KCl, 3.0 $MgCl_2$, 0.05 $CdCl_2$, 0.001 tetrodotoxin, 10 glucose and 10 HEPES (pH adjusted to 7.2 with TrisCl).

The composition (in mM) of the electrode solutions for the different recording conditions are listed as follows:

1. Na^+ and Ba^{2+} currents: 70 CsCl, 70 Cs-glutamate, 2 $MgCl_2$, 10 HEPES (pH adjusted to 7.2 with TrisCl).

2. K^+ currents: 120 K-aspartate, 20 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES (pH adjusted to 7.2 with TrisCl).

All reported membrane potentials for Na^+ currents were corrected for a liquid junction potential between the pipette and bath solution of +10 mV (Barry, 1994). The junction potentials for K^+ and Ba^{2+} current recordings were less than 3 mV and were not corrected for. Exponential fits were performed with the Clampfit program. All values in the text are reported as mean ± SEM. Differences between groups were considered to be significant when $P < 0.05$ using paired *t* test or ANOVA, followed by Fisher's LSD test. The potencies of inhibitors were estimated by determining the ED_{50} values of the dose-response curves using a four-parameter logistic curve-fitting program (ALLFIT; DeLean *et al.*, 1978).

Drugs

To prevent oxidation, dopamine, quinpirole (dopamine D_2 agonist) and SKF 38393 (dopamine D_1 agonist) were prepared immediately prior to each experiment in 0.05 M acetic acid and stored under argon throughout the duration of the experiment. Stock solutions of spiperone (dopamine D_2 antagonist), SKF-83566 (dopamine D_1 antagonist) and (±)-Bay K 8644 (Ca^{2+} channel activator) and nifedipine (Ca^{2+} channel antagonist) were prepared in ethanol. Apomorphine (general dopamine receptor agonist) was dissolved in DMSO. Tetrodotoxin citrate was dissolved in saline. Aliquots of stock solutions were stored at -20 °C until use, when they were diluted to final concentrations in saline. The highest concentration of ethanol, DMSO or acetic acid was ≤ 0.1%, which had no effect on either basal hormone release or ionic currents. All drugs were purchased from Research Biochemicals Incorporated (Natick, MA, USA).

RESULTS

Effects of dopamine-D_2 receptor activation on voltage-dependent Ca^{2+} channel-mediated GTH-II secretion

To activate voltage-dependent Ca^{2+} channels, high concentrations of extracellular K^+ or the Ca^{2+} channel activator, Bay K 8644, were used. Application of 30 mM K^+ stimulated GTH-II secretion from goldfish pituitary cells in perifusion experiments (Fig. 5.1). To determine whether K^+-stimulated GTH-II secretion is dependent on extracellular Ca^{2+}-influx through voltage-dependent Ca^{2+} channels, the effects of extracellular Ca^{2+} removal and the voltage-dependent Ca^{2+} channel blocker, nifedipine, were examined. Removal of extracellular Ca^{2+} reduced the net GTH-II response to a 5-min pulse of 30 mM K^+ from 285.7 ± 69.7% to 92.3% ($P < 0.05$, paired *t*-test, $n = 4$). Similarly, application of 10 μM nifedipine reduced the net GTH-II response to K^+ from 141.0 ± 16.6% to 37.5 ± 1.7% ($P < 0.05$, paired *t*-test, $n = 4$). Furthermore, 10 μM Bay K 8644, stimulated GTH-II secretion in 2-hr static incubation experiments (Fig. 5.2). These data indicate that activation of Ca^{2+} influx through voltage-dependent Ca^{2+} channels can stimulate short-term and long-term GTH-II secretion.

To examine the actions of dopamine on voltage-dependent Ca^{2+} channel-mediated GTH-II secretion, dopamine receptor agonists and antagonists were used. Addition of 100 nM dopamine reduced K^+-stimulated GTH-II secretion in perifusion experiments (Fig. 5.1A). Similarly, 100 nM dopamine reduced 10 μM Bay K 8644-stimulated GTH-II secretion in static culture experiments (Fig. 5.2A). Application of 100 nM quinpirole, a dopamine D_2 receptor agonist, mimicked the actions of dopamine, reducing both 30 mM KCl- and 10 μM Bay K 8644-stimulated GTH-II secretion in perifusion (Fig. 5.1B) and static culture (Fig. 5.2B) experiments, respectively. Additionally, the inhibitory actions of dopamine on Bay-K 8644-stimulated GTH-II secretion were blocked by the application of spiperone, a dopamine D_2-receptor antagonist (Fig. 5.2C). Spiperone alone did not affect either basal ($P > 0.05$, paired *t*-test, $n = 10$) or Bay K 8644-stimulated ($P > 0.05$, paired *t*-test, $n = 10$) GTH-II secretion. These data indicate that dopamine D_2-receptor activation reduces the secretion of GTH-II mediated by Ca^{2+} entry through voltage-dependent Ca^{2+} channels.

Dopamine receptor activation does not affect Ca^{2+}-induced GTH-II secretion

Dopamine may act on Ca^{2+}-dependent signaling events down-stream of Ca^{2+} entry through voltage-dependent Ca^{2+} channels to inhibit GTH-II secretion. To test this hypothesis, the effects of the general dopamine agonist, apomorphine, on Ca^{2+}-ionophore-stimulated GTH-II secretion were examined. The Ca^{2+} ionophore, A23187, stimulates GTH-II secretion by inducing extracellular Ca^{2+} entry independent of voltage-dependent Ca^{2+} channels. Application of 1 µM apomorphine did not affect GTH-II secretion induced by 10 µM A23187 (Fig. 5.3). Thus, it is unlikely that dopamine receptor activation affects GTH-II secretion down-stream of Ca^{2+} entry.

Dopamine-induced reduction in Ba^{2+} current amplitude through voltage-dependent Ca^{2+} channels

To directly elucidate the effects of dopamine on voltage-dependent Ca^{2+} channels in goldfish gonadotrophs, perforated-patch voltage-clamp recordings were performed. In the present study, Ba^{2+} was used as the charge carrier instead of Ca^{2+} for four reasons: 1. to minimize current rundown commonly associated with Ca^{2+} currents; 2. Ba^{2+} normally permeates more readily than Ca^{2+} through Ca^{2+} channels; 3. Ba^{2+} is relatively impermeant through Na^{+} channels; and 4. Ba^{2+} blocks outward K^{+} currents (Bean, 1992). In previous studies, the activation and inactivation properties of the currents carried by Ca^{2+} and Ba^{2+} were demonstrated to be similar in identified goldfish gonadotrophs (Chapter 3). In the present study, command potentials more depolarized than -30 mV elicited an inward Ba^{2+} current that reached a maximum amplitude between 10 and 20 mV and inactivated slowly during the 200 msec command pulse (Fig. 5.4 and 5.6). The maximal Ba^{2+} current amplitude under control conditions was 112 ± 8 pA (mean ± SEM; range 36 to 368 pA, $n = 48$). The voltage-dependent Ba^{2+} currents recorded in the present study were similar to the high-voltage activated, dihydropyridine-sensitive Ca^{2+} currents previously characterized in goldfish (Chapter 3) and mammalian gonadotrophs (Stojilkovic and Catt, 1992; Tse and Hille, 1993).

Dopamine caused a dose-dependent reduction in the Ba^{2+} current amplitude with an ED_{50} of 0.97 ± 0.34 nM (Fig. 5.4A). A maximal decrease in Ba^{2+} current amplitude of 45.2 ± 2.7 % was observed with 1 μM dopamine. Maximal inhibition was observed within 30 sec of dopamine application and the response to dopamine was reversible in all gonadotrophs studied (Fig. 5.4B). Dopamine (100 nM) reduced both initial and prolonged Ba^{2+} current amplitudes (Fig. 5.4C), but did not shift the activation threshold, current-voltage relationship or the apparent reversal potential of the current (Fig. 5.4D). The reduction in maximal Ba^{2+} current amplitude by 100 nM dopamine was more pronounced during the initial (0 to 25 msec) inward current than during the prolonged (175 to 200 msec) inward current (46.4 ± 4.3 % reduction in initial current vs. 37.7 ± 5.9 % reduction in prolonged current; $P < 0.05$, paired t test, $n = 4$).

The inhibitory effects of dopamine on Ba^{2+} currents in goldfish gonadotrophs appeared to be specific. Serotonin, another biogenic amine which has also been shown to directly regulate GTH-II secretion from goldfish gonadotrophs (Wong *et al.*, 1996), did not affect the Ba^{2+} current amplitude ($n = 3$; data not shown). Furthermore, in identified goldfish somatotrophs, 100 nM dopamine increased the Ba^{2+} current amplitude to 149 ± 16% of control values ($n = 3$). These results indicate that dopamine does not cause a non-specific inhibition of Ba^{2+} currents in goldfish pituitary cells.

Apomorphine effects on voltage-dependent Ba^{2+} currents

Apomorphine, a general dopamine receptor agonist, mimicked the actions of dopamine on voltage-dependent Ca^{2+} channels in identified goldfish gonadotrophs. Application of 1 μM apomorphine reduced the Ba^{2+} current amplitude by 54.5 ± 12.4% compared to vehicle controls (Fig. 5.5A; $P < 0.05$ *vs.*

zero change, one-group *t*-test, $n = 3$). Following washout, the Ba^{2+} current amplitude returned to 81.5 ± 2.5% of control levels. Apomorphine did not appear to shift the activation threshold or consistently shift the current-voltage relationship of the voltage-dependent Ba^{2+} current (Fig. 5.5B). These results are consistent with the inhibitory actions of dopamine receptor activation on voltage-dependent Ba^{2+} currents in goldfish gonadotrophs.

Activation of dopamine D_2 receptors reduces Ba^{2+} current amplitude

The effects of dopamine-D_1 and -D_2 receptor agonists on voltage-dependent Ba^{2+} currents were monitored to determine the dopamine receptor subtype mediating the actions of dopamine. Quinpirole, a dopamine D_2 agonist, mimicked the action of dopamine. Quinpirole caused a dose-dependent reduction in the Ba^{2+} current amplitude with an ED_{50} of 2.73 ± 1.38 nM (Fig. 5.6A). A maximal decrease in Ba^{2+} current amplitude of 43.2 ± 9.8 % was observed with 1 μM quinpirole ($n = 4$). The magnitude of the maximal suppression of Ba^{2+} current amplitude induced by dopamine and quinpirole were not significantly different from one another ($P > 0.05$, unpaired *t*-test). Quinpirole (100 nM) reduced both initial and prolonged Ba^{2+} current amplitudes, but did not shift the activation threshold, current-voltage relationship or the apparent reversal potential of the current ($n = 12$; Fig 5.6B, C). Additionally, when the Ba^{2+} currents in the absence or presence of 100 nM quinpirole were normalized to their maximal amplitudes and plotted against membrane potential, their was no change in the membrane potential corresponding to half-maximal activation ($E_{1/2}$) of the currents (Table 5.1). Thus, quinpirole did not modify the voltage-dependence of activation. Following the removal of 100 nM quinpirole, the maximal Ba^{2+} current amplitude returned to 92.7 ± 3.2% of the control values ($n = 12$). Unlike quinpirole, 100 nM SKF 38393, a dopamine-D_1 receptor agonist, did not affect the amplitude or shift the current-voltage relationship of the Ba^{2+} current (Fig. 5.7).

The receptor subtype mediating the inhibitory actions of dopamine on voltage-dependent Ba^{2+} currents was further examined with dopamine D_1- and D_2-receptor antagonists. Application of 1 μM SKF 83566, a dopamine-D_1 receptor antagonist, did not affect the inhibitory action of 10 nM dopamine on Ba^{2+} current amplitude (Fig. 5.8A). Conversely, the dopamine-D_2 receptor antagonist, spiperone (1 μM), blocked the inhibitory action of 10 nM dopamine (Fig. 5.8B). Neither spiperone nor SKF-83566 alone affected voltage-dependent Ba^{2+} currents. Taken together, these results indicate that dopamine inhibition of Ba^{2+} currents is mediated by D_2-like receptors.

Quinpirole affects the activation and inactivation kinetics, as well as the steady-state inactivation properties of Ba^{2+} currents

To monitor the effects of quinpirole on the activation kinetics of Ba^{2+} currents in identified goldfish gonadotrophs, the time to peak current was determined during a 40 msec voltage step to +10 mV (holding potential = -80 mV) in the presence or absence of quinpirole (Fig. 5.9A). Quinpirole (100 nM) increased the time to peak current, indicating that quinpirole slowed the activation time course of Ba^{2+} currents. In addition to affecting the activation kinetics, 100 nM quinpirole also slowed the inactivation kinetics of the voltage-dependent Ba^{2+} current (Fig. 5.9B). In seven representative cells, the inactivating phase of the Ba^{2+} current was best approximated using a single exponential fit. In the example shown (Fig. 5.9B; left panel), the time constant (τ) of inactivation was increased from 107.9 msec to 165.8 msec in the presence of 100 nM quinpirole. The effect of quinpirole on the inactivation kinetics was also demonstrated by comparing the initial and steady-state (490 - 500 msec) Ba^{2+} current amplitude elicited by a 500 msec voltage-step to +10 mV or +20 mV from a holding potential of -80 mV (Table 5.2). Under control conditions, the initial Ba^{2+} current amplitude was significantly greater than the steady-state current amplitude. Conversely, in the presence of 100 nM quinpirole, the initial and steady-state Ba^{2+} current amplitudes were similar. These results indicate that quinpirole either slows or removes the inactivating component of the voltage-dependent Ba^{2+} current in goldfish gonadotrophs.

To determine if dopamine-D_2 receptor activation affects the steady-state inactivation properties of the voltage-dependent Ba^{2+} current, a two-pulse protocol was used (Bezanilla and Armstrong, 1977). Gonadotrophs held at -80 mV were subjected to pre-pulse potentials ranging from -90 to +20 mV for 500 msec, after which a 40 msec test pulse to +10 mV was applied (Fig 5.10). Whereas steady-state inactivation was observed under control conditions, no steady-state inactivation of the voltage-dependent Ba^{2+} current was observed in the presence of 100 nM quinpirole (Fig. 5.10).

Voltage-dependent K^+ and Na^+ currents were not affected by dopamine

In frog melanotrophs (Valentijn *et al.*, 1991), dopamine alters voltage-dependent K^+ and Na^+ current amplitude to decrease membrane excitability and thereby indirectly impair Ca^{2+} channel gating. To examine this possibility in identified goldfish gonadotrophs, the actions of dopamine on voltage-dependent K^+ and Na^+ currents were monitored in perforated patch-clamp experiments. Dopamine did not affect current-amplitude or shift the current-voltage relationship of either K^+ or Na^+ currents (Fig. 5.11). Similarly, quinpirole did not affect voltage-dependent K^+ current amplitude

(K^+ current amplitude elicited by a 50 msec voltage-step to +20 mV from a holding potential of -80 mV: control, 478.3 ± 52.7 pA; 1 µM quinpirole, 471. 7 ± 50.5 pA; washout, 521.5 ± 12.5 pA; $P > 0.05$; $n = 3$). Therefore, in contrast to voltage-dependent Ba^{2+} currents, neither voltage-dependent K^+ nor Na^+ currents were affected by dopamine-D_2 receptor activation.

DISCUSSION

Results of the present study clearly demonstrate that dopamine reduces Ba^{2+} current influx through high-voltage-activated Ca^{2+} channels in identified goldfish gonadotrophs. The reduction in Ba^{2+} current amplitude was dose-dependent and reversible. Moreover, the dopamine-induced reduction in Ba^{2+} current amplitude was not caused by a non-specific inhibition of voltage-dependent Ba^{2+} currents, as dopamine increased Ba^{2+} current amplitude in identified goldfish somatotrophs. This latter response is consistent with the involvement of voltage-dependent Ca^{2+} channels in mediating dopamine-stimulated growth hormone secretion in goldfish (Chang and de Leeuw, 1990; Wong *et al.*, 1994a).

Three lines of evidence suggest that the inhibitory action of dopamine on voltage-dependent Ba^{2+} currents in goldfish gonadotrophs is mediated by dopamine D_2-like receptors. Firstly, the D_2 agonist quinpirole mimicked the inhibitory action of dopamine on voltage-dependent Ba^{2+} currents with a similar potency and efficacy. Secondly, the dopamine D_1-receptor agonist, SKF 38393, did not mimic the actions of dopamine on voltage-dependent Ba^{2+} currents. Thirdly, the inhibitory actions of dopamine on voltage-dependent Ba^{2+} currents were blocked by the dopamine D_2-receptor antagonist, spiperone, whereas the dopamine D_1-receptor antagonist, SKF-83566, had no effect. Activation of dopamine D_2-like receptors has also been linked to the inhibition of voltage-dependent Ca^{2+} currents in rat lactotrophs (Lledo *et al.*, 1990), rat melanotrophs (Keja *et al.*, 1992) and frog melanotrophs (Valentijn *et al.*, 1991).

Agonist-induced suppression of Ca^{2+} current through voltage-dependent Ca^{2+} channels can occur by a decrease in channel density or conductance, as well as by a shift in the voltage-dependence of channel gating (Marchetti *et al.*, 1986; Bean, 1989; Elmslie *et al.*, 1990; Boland and Bean, 1993). In this regard, the present study provides some clues as to the possible mechanisms mediating the dopamine-induced reduction in Ba^{2+} current amplitude in goldfish gonadotrophs. Quinpirole reversibly slowed down Ca^{2+} channel activation, an effect that may be attributed to the removal of a fast-activating subtype of high-voltage activated channels. In support of this possibility, dihydropyridine Ca^{2+} channel antagonists reduced the Ba^{2+} current in goldfish gonadotrophs by only 39% (Chapter 3), suggesting that multiple high-voltage activated channel subtypes may be present. Alternatively, dopamine may induce a fraction of the channels to shift from a "willing" to a "reluctant" gating mode, in which channel opening requires stronger membrane depolarization (Bean, 1989). The persistent reduction in Ba^{2+} current amplitude in response to quinpirole during prolonged (200 to 500 msec) membrane depolarization suggests that other inhibitory mechanisms may reduced Ca^{2+} channel conductance independent of voltage changes. Further studies are required to elucidate the mechanisms involved in dopamine-induced reduction in current amplitude through voltage-dependent Ca^{2+} channels.

In addition to altering the kinetics of channel activation, quinpirole altered the inactivation properties of the voltage-dependent Ba^{2+} current in goldfish gonadotrophs. In the presence of quinpirole, the time course of Ba^{2+} current inactivation was slowed. In addition, application of quinpirole removed the steady-state inactivation property of the Ba^{2+} current. These results suggest that a component of the total Ba^{2+} current is abolished by the activation of dopamine D_2-like receptors. Whether this reflects the inhibition of one or several Ca^{2+} channel subtypes, or a change in the kinetic properties of a single channel type is not clear. However, as previously mentioned, the high-voltage activated Ca^{2+} channels observed in goldfish gonadotrophs exhibited only a partial sensitivity to dihydropyridine-blockade (Chapter 3), which suggests the presence of multiple channel subtypes. Also, in rat melanotrophs and rat lactotrophs, dopamine differentially affected multiple voltage-dependent Ca^{2+} channel subtypes (Keja *et al.*, 1992; Lledo *et al.*, 1992).

Consistent with the inhibitory action of dopamine on voltage dependent Ba^{2+} currents, dopamine and its D_2 agonist, quinpirole, attenuated the GTH-II release response to activators of voltage-dependent Ca^{2+} channels. Dopamine and quinpirole attenuated acute GTH-II secretion stimulated by high concentrations of extracellular K^+ in perifusion experiments. High concentrations of extracellular K^+ induce membrane depolarization, resulting in the subsequent activation of voltage-dependent Ca^{2+} channels. The involvement of voltage-dependent Ca^{2+} channels during K^+-stimulated GTH-II secretion and increases in $[Ca^{2+}]_i$ in goldfish was confirmed in this and other studies (Jobin and Chang, 1992b). Dopamine and quinpirole inhibited prolonged GTH-II secretion stimulated by the dihydropyridine-sensitive Ca^{2+} channel activator, Bay K 8644. Additionally, the inhibitory actions of dopamine on Bay K 8644-stimulated GTH-II secretion were blocked by the dopamine D_2-receptor antagonist, spiperone. Activation of dopamine D_2 receptors, however, did not affect Ca^{2+} ionophore-induced GTH-II secretion from goldfish pituitary cells in this or previous studies (Chang *et al.*, 1993). Taken together, these results indicate that dopamine D_2 receptor-mediated inhibition of GTH-II secretion is due to the inhibition of voltage-dependent Ca^{2+} channels, rather than inhibition of Ca^{2+} dependent signaling events downstream of Ca^{2+} entry.

Dopamine D_2-receptor activation in frog melanotrophs augments voltage-dependent K^+ currents and attenuates voltage-dependent Na^+ currents to reduce plasma membrane electrical activity, resulting in a reduction in Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Valentijn *et al.*, 1993). Dopamine also augments voltage-dependent K^+ currents in rat lactotrophs (Lledo *et al.*, 1990). In the present study, dopamine D_2 receptor activation did not modulate voltage-dependent K^+ or Na^+ currents in goldfish gonadotrophs. The lack of effect of dopamine on voltage-dependent Na^+ currents was not unexpected, as voltage-dependent Na^+ channels are not involved in basal or GnRH-stimulated GTH-II secretion (Chapter 4). Somewhat surprising was the lack of effect of dopamine on voltage-dependent K^+ currents in goldfish gonadotrophs, although in rat melanotrophs dopamine also has no affect on voltage-dependent K^+ currents (Keja *et al.*, 1992). However, the present study does not eliminate the possible activation of non-voltage-dependent K^+ currents, as demonstrated in rat lactotrophs (Einhorn and Oxford, 1993), rat melanotrophs (Stack and Surprenant, 1991) and frog melanotrophs (Valentijn *et al.*, 1993).

Dopamine inhibition of GnRH-induced GTH-II secretion *in vivo* and *in vitro* are mediated by D_2 receptors (Peter *et al.*, 1986; Chang *et al.*, 1993). The ED_{50} for quinpirole-induced inhibition of sGnRH and cGnRH-II stimulated GTH-II secretion is 1.0 ± 0.7 nM and 0.2 ± 0.1 nM, respectively (calculated from Chang *et al.*, 1990b). These values are similar to those observed for quinpirole-induced inhibition of Ba^{2+} currents in this study. The similar potencies of quinpirole actions on GnRH-stimulated GTH-II secretion and Ba^{2+} currents further suggest that similar receptors are involved. Thus, the finding that dopamine D_2-receptor activation inhibits voltage-dependent Ca^{2+} channels likely has important implications concerning the neuroendocrine regulation of gonadotroph cell function in goldfish. Extracellular Ca^{2+} influx through voltage-dependent Ca^{2+} channels is a critical event in mediating GnRH-induced GTH-II secretion from goldfish gonadotrophs. Removal of extracellular Ca^{2+} or the addition of inorganic and organic voltage-dependent Ca^{2+} channel blockers reduces GnRH-stimulated GTH-II secretion (Chang *et al.*, 1993, 1996). Consequently, a reduction in Ca^{2+} channel activity by dopamine D_2-receptor activation would impair GnRH-stimulated GTH-II secretion. As dopamine does not affect Ca^{2+} signaling events down stream of Ca^{2+} entry, the present data suggest that dopamine inhibits GnRH-stimulated GTH-II secretion primarily by reducing Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

It is not yet known if dopamine D_2-receptors in goldfish are coupled to voltage-dependent Ca^{2+} channels via G-proteins, as in other pituitary cells (Stack and Surprenant, 1991; Lledo *et al.*, 1992; Valentijn *et al.*, 1993), or if they are indirectly coupled through other second messenger pathways. It will be of interest to investigate whether the short term actions of dopamine on the activation kinetics of Ca^{2+} channels are mediated by G-protein coupling between dopamine D_2 receptors and channel proteins as demonstrated to occur in frog melanotrophs (Valentijn *et al.*, 1993). Alternatively, the prolonged inhibition of Ba^{2+} currents observed in the present study may be due to other second messenger systems coupled to Ca^{2+} channels. One possibility is that dopamine may interact with PKC to reduce Ca^{2+} channel activity. In previous studies, dopamine has been demonstrated to reduce PKC-stimulated increases in $[Ca^{2+}]_i$ and GTH-secretion (Jobin and Chang, 1992b; Jobin and Chang, 1993; Chang *et al.*, 1993).

In summary, dopamine acts via dopamine D_2-like receptors to reduce Ca^{2+} influx through voltage-dependent Ca^{2+} channels, resulting in a reduction in GTH-II-secretion (Fig. 5.12). Unlike other pituitary cell types, voltage-dependent Na^+ and K^+ channels do not appear to be affected by dopamine receptor activation. These studies suggest that dopamine-induced inhibition of voltage-dependent Ca^{2+} channels is a critical event in modulating GnRH-stimulated GTH-II secretion. The specific transduction pathways mediating the actions of dopamine, as well as the interactions between dopamine and GnRH on membrane excitability, remain to elucidated.

Table 5.1. Effects of quinpirole on the voltage-dependent activation curve of Ba^{2+} currents in identified goldfish gonadotrophs.

Activation properties[a]	Control	100 nM Quinpirole
$E_{1/2}$ (mV)	-10.6 ± 1.4	-8.2 ± 1.5[b]
k	-4.9 ± 0.2	-5.5 ± 0.3[b]

[a] The activation curve of the voltage-dependent Ba^{2+} current was fit with a single Boltzmann relation, $I/Imax = Imax/\{1 + \exp[(E-E_{1/2})/k]\}$

[b] Within the same group, values which are similar to controls ($P > 0.05$, paired *t*-test, $n = 12$) are identified by the same superscript.

Table 5.2. Effects of 100 nM quinpirole on peak (10 - 20 msec) and steady-state (480 - 500 msec) voltage-dependent Ba^{2+} currents in identified goldfish gonadotrophs.[a]

Command potential (mV)	Control (pA)		100 nM Quinpirole (pA)	
	Peak	Steady-state	Peak	Steady-state
+10	-127.7 ± 15.2	-110.1 ± 16.4[b]	-66.4 ± 6.1	-72.6 ± 3.9
+20	-110.1 ± 13.8	-88.3 ± 12.9[b]	-62.4 ± 5.8	-57.9 ± 6.1

[a] The membrane potential was stepped from -80 mV to the command potential indicated for 500 msec.

[b] Significantly different from peak current amplitude within each group ($P < 0.05$, paired *t* -test; $n = 7$).

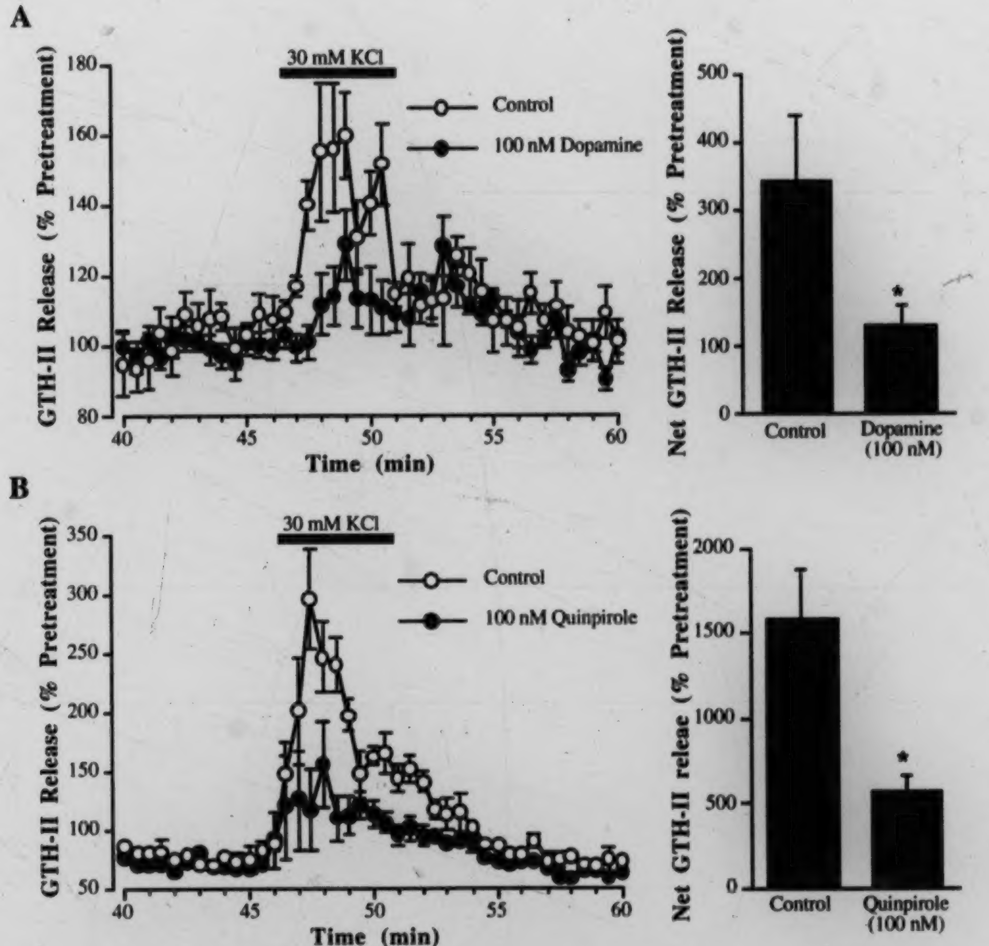


Fig. 5.1. Effects of dopamine and quinpirole on K$^+$-stimulated GTH-II secretion from dispersed goldfish pituitary cells in perifusion experiments. Left panel: hormone release response to a 5-min pulse of 30 mM K$^+$ (*bar*) in the presence (*filled circles*) or absence (*open circles*) of 100 nM dopamine (A) or its D$_2$ agonist quinpirole (B). Right panel: net GTH-II response to 30 mM K$^+$ in the presence or absence of 100 nM dopamine (A) or quinpirole (B). GTH-II release responses (mean ± SEM; $n = 4$ in control groups and 6 in treatment groups) were normalized as a percentage of the pretreatment values (17.1 ± 1.0 ng/ml/2 x 10^6 cells and 22.0 ± 3.5 ng/ml/2 x 10^6 cells, in A and B, respectively). *Asterisk* denotes significant difference from control ($P <$ 0.05, unpaired *t* -test).

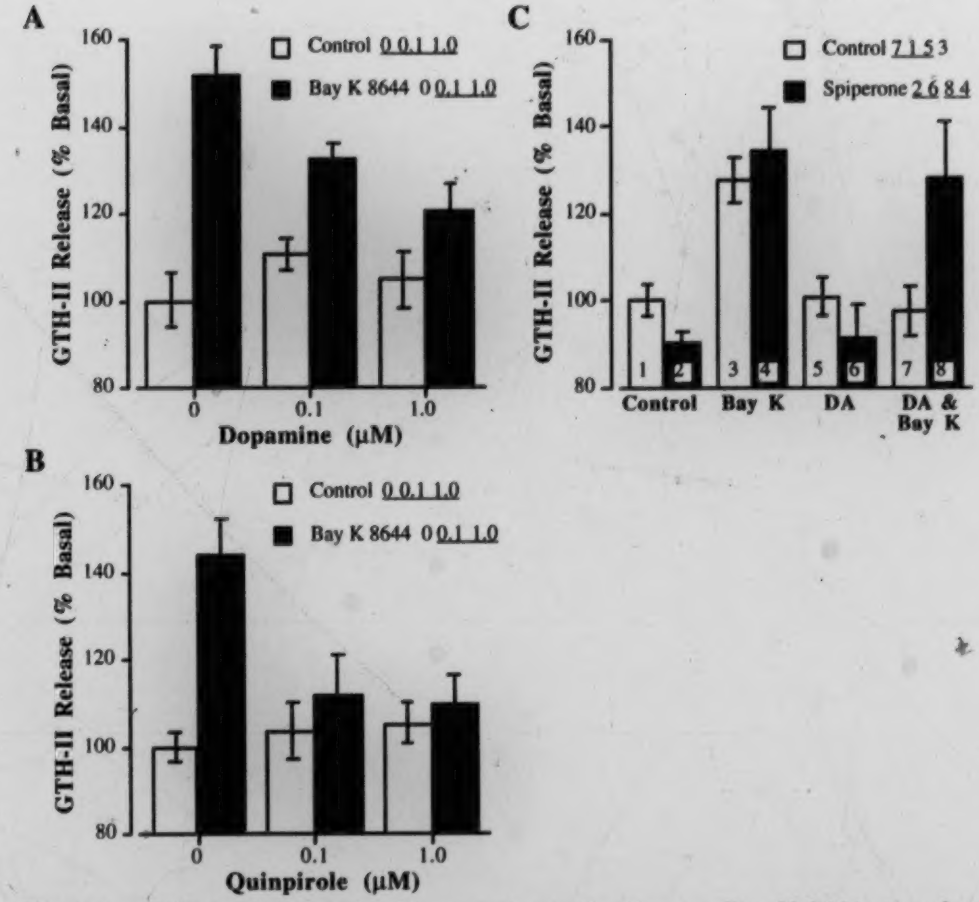


Fig. 5.2. Effects of dopamine D_2-receptor activation on Bay K 8644-stimulated GTH-II secretion from dispersed goldfish pituitary cells in static incubation experiments. A and B. Effects of 100 nM dopamine (A; n = 8 to 16) and 100 nM quinpirole (B; n = 14) on 10 µM Bay K 8644-stimulated GTH-II secretion. C Effects of 10 µM spiperone on 1 µM dopamine (DA)-induced inhibition of 10 µM Bay K 8644 (Bay K)-stimulated GTH-II secretion (n = 10). Results were normalized to % basal GTH-II secretion (247 ± 27.7 ng/ml/0.25 x 10^6 cells) and are presented as mean ± SEM. Treatment groups having similar GTH-II responses (ANOVA followed by Fisher's LSD test $P > 0.05$) are identified by the same *underscore*.

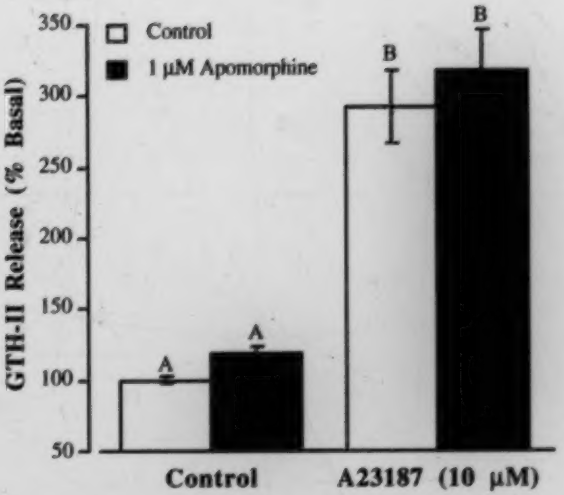
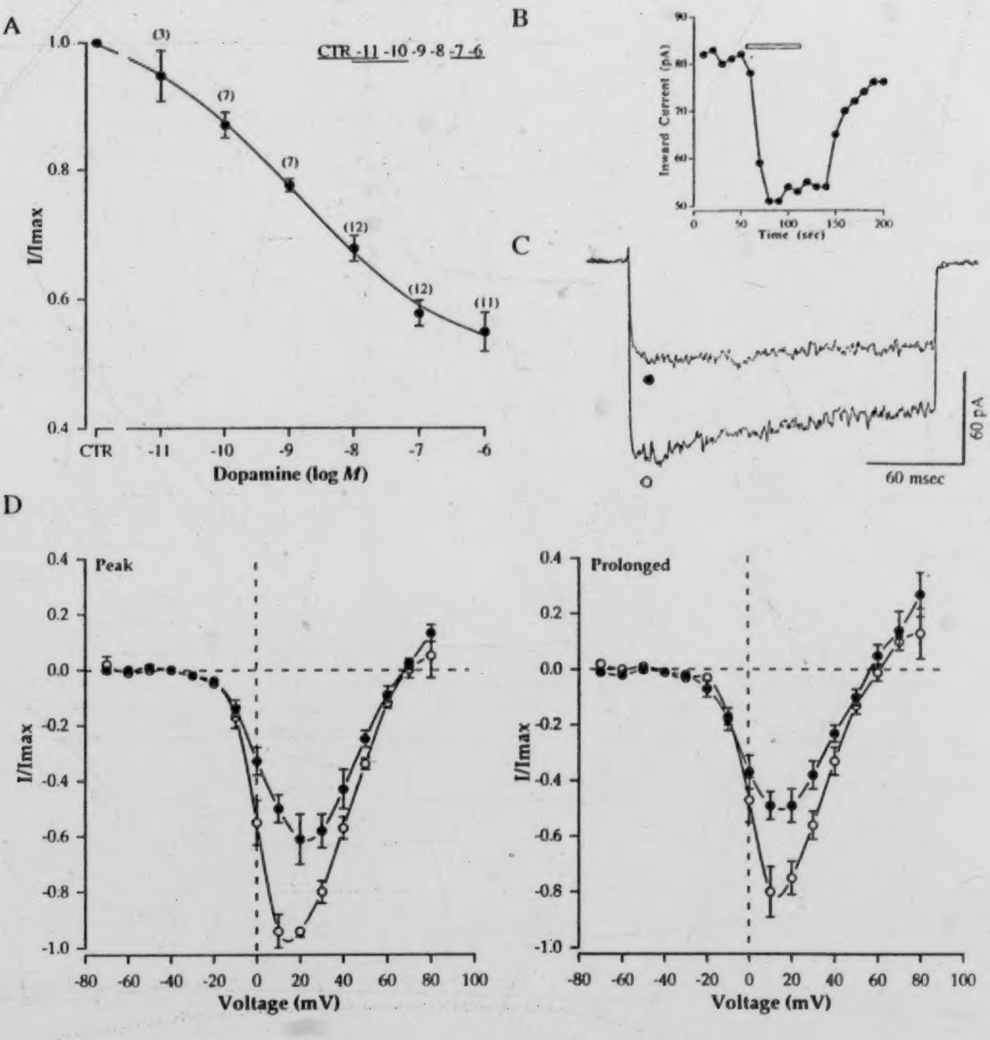


Fig. 5.3. Effects of apomorphine on A23187-stimulated GTH-II secretion from dispersed goldfish pituitary cells in static incubation experiments. Results were normalized to % basal GTH-II secretion (225.7± 30.7 ng/ml/0.25 x 10^6 cells; mean ± SEM, n = 16). Treatment groups having similar GTH-II responses (ANOVA followed by Fisher's LSD test $P > 0.05$) are identified by the same letter.

Fig. 5.4. Effects of dopamine on voltage-dependent Ba^{2+} currents in identified goldfish gonadotrophs All currents in A – C were elicited by a 40-msec voltage-pulse to +10 mV from a holding potential of -80 mV. A. Dopamine induced a dose-dependent inhibition of Ba^{2+} currents; n = 3 to 12 (*brackets*). Underscores denote values which are similar (ANOVA and Fisher's LSD test, $P > 0.05$). B. Time course of 100 nM dopamine-induced inhibition of voltage-dependent Ba^{2+} currents. Duration of dopamine application is indicated by the open horizontal bar. Result is representative of 12 similar experiments. C. Superimposed Ba^{2+} current traces from a representative gonadotroph in the presence (*filled circle*) or absence (*open circle*) of 100 nM dopamine. D. Current-voltage relationship of the initial (0 to 25 msec) and prolonged (175 to 200 msec) voltage-dependent Ba^{2+} currents evoked by 200-msec voltage-steps to the potentials indicated (holding potential = -80 mV) in the presence (*filled circles*) or absence (*open circles*) of 100 nM dopamine (n = 4). All currents were normalized to the maximal inward current and are presented as mean ± SEM.


A
1.0
0.8
0.6
0.4
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(3)
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(7)
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(11)
CTR -11 -10 -9 -8 -7 -6
CTR
-11
-10
-9
-8
-7
-6
Dopamine (log M)
B
90
80
70
60
50
Inward Current (pA)
0
50
100
150
200
Time (sec)
C
●
○
60 pA
60 msec
D
Peak
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
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I/Imax
-80
-60
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-20
0
20
40
60
80
100
Voltage (mV)
Prolonged
0.4
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-0.6
-0.8
-1.0
I/Imax
-80
-60
-40
-20
0
20
40
60
80
100
Voltage (mV)

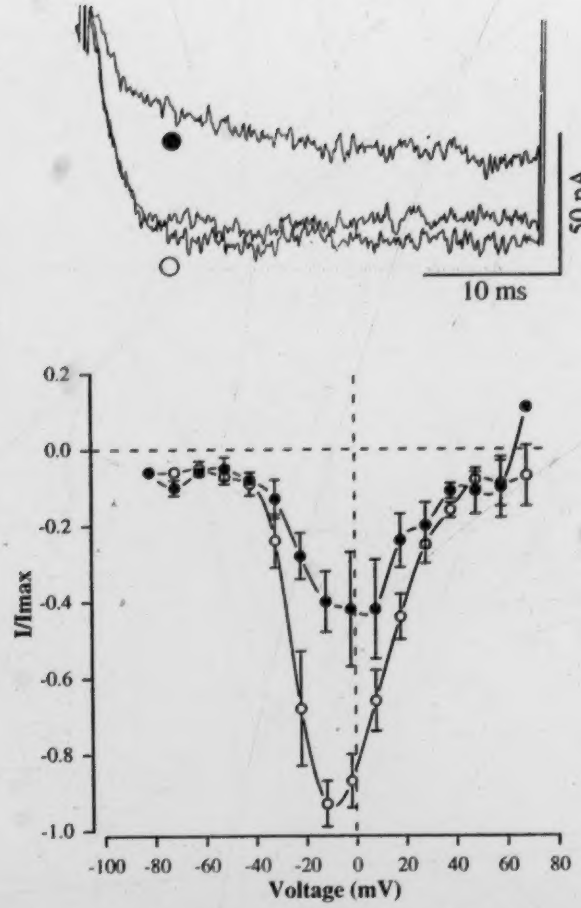


Fig. 5.5. Effects of apomorphine on voltage-dependent Ba^{2+} currents in identified goldfish gonadotrophs. A. Tight-seal whole-cell, voltage-clamp recordings of Ba^{2+} currents elicited during a 40 msec voltage-step to +0 mV before (*open circles*), during (*closed circles*), and after (*diamond*) the application of 1 µM apomorphine (holding potential = -80 mV). B. Current-voltage relationship of the voltage-dependent Ba^{2+} currents evoked by 200-msec voltage-steps to the potentials indicated (holding potential = -80 mV) in the presence (*filled circles*) or absence (*open circles*) of 1 µM apomorphine (*n* = 3). Tight-seal whole-cell, patch-clamp recordings were performed as previously described in Chapter 3. The pipette solution was composed of (in mM); 120 Cs-glutamate, 20 TEA, 2 $MgCl_2$, 11 EGTA, 1 $CaCl_2$, 2.5 Mg-ATP, 0.2 Na-GTP (pH; 7.2). The external solution was the same as that used in perforated-patch, whole-cell recordings. All voltages have been corrected for a liquid junction potential of 12 mV as previously described (Chapter 3). Currents were normalized to maximal inward current (mean ± SEM).

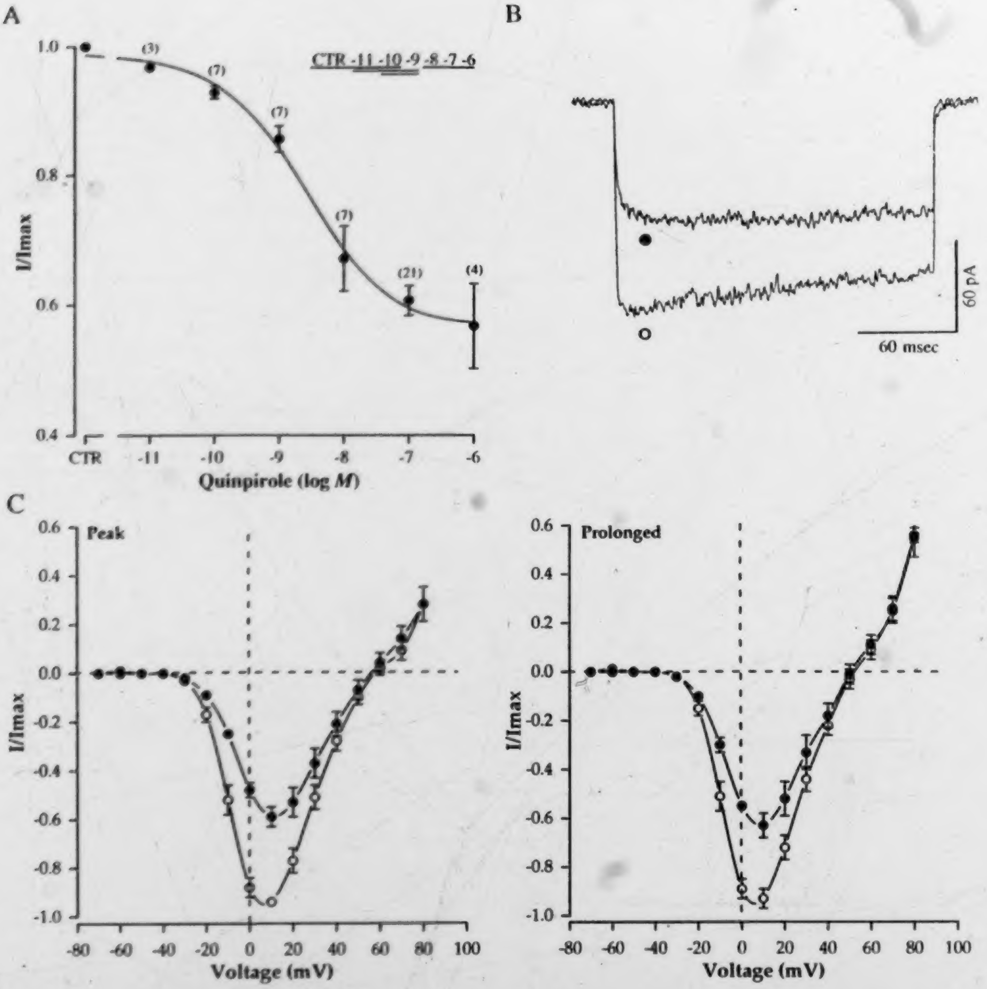


Fig. 5.6. Effects of quinpirole on voltage-dependent Ba^{2+} currents in identified goldfish gonadotrophs. All currents in A and B were elicited by a 40-msec voltage-step to +10 mV from a holding potential of -80 mV. A. Quinpirole induced a dose-dependent inhibition of Ba^{2+} currents; n = 3 to 21 (*brackets*). Underscores denote values which are similar (ANOVA and Fisher's LSD test, $P > 0.05$). B. Superimposed Ba^{2+} current traces from a representative gonadotroph in the presence (*filled circle*) or absence (*open circle*) of 100 nM quinpirole. C. Current-voltage relationship of the peak (10 to 20 msec) and steady-state (180 to 200 msec) voltage-dependent Ba^{2+} current evoked by 200-msec voltage-steps to the potentials indicated (holding potential = -80 mV), in the presence (*filled circles*) or absence (*open circles*) of 100 nM quinpirole (n = 12). All currents were normalized to the maximal inward current and are presented as mean ± SEM.

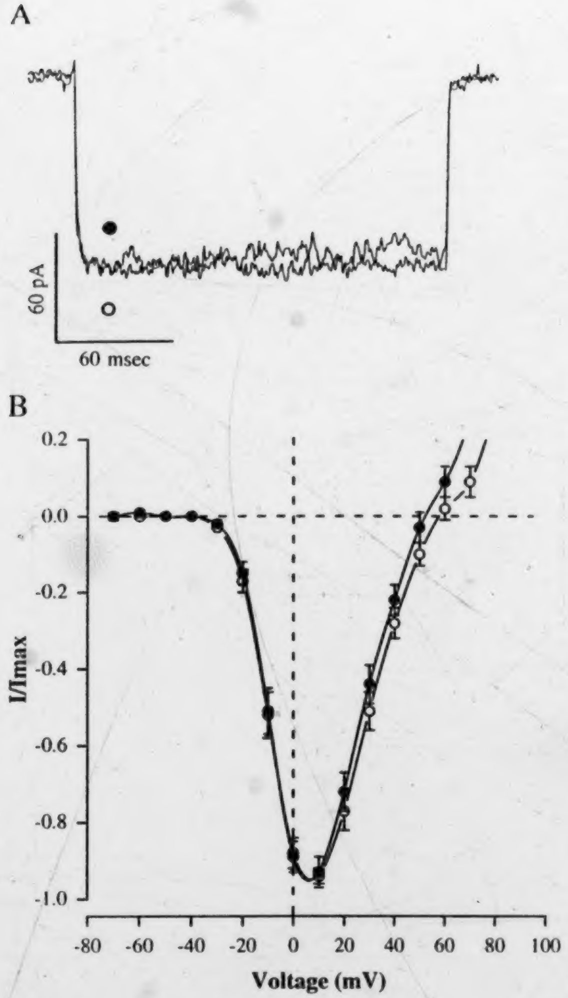
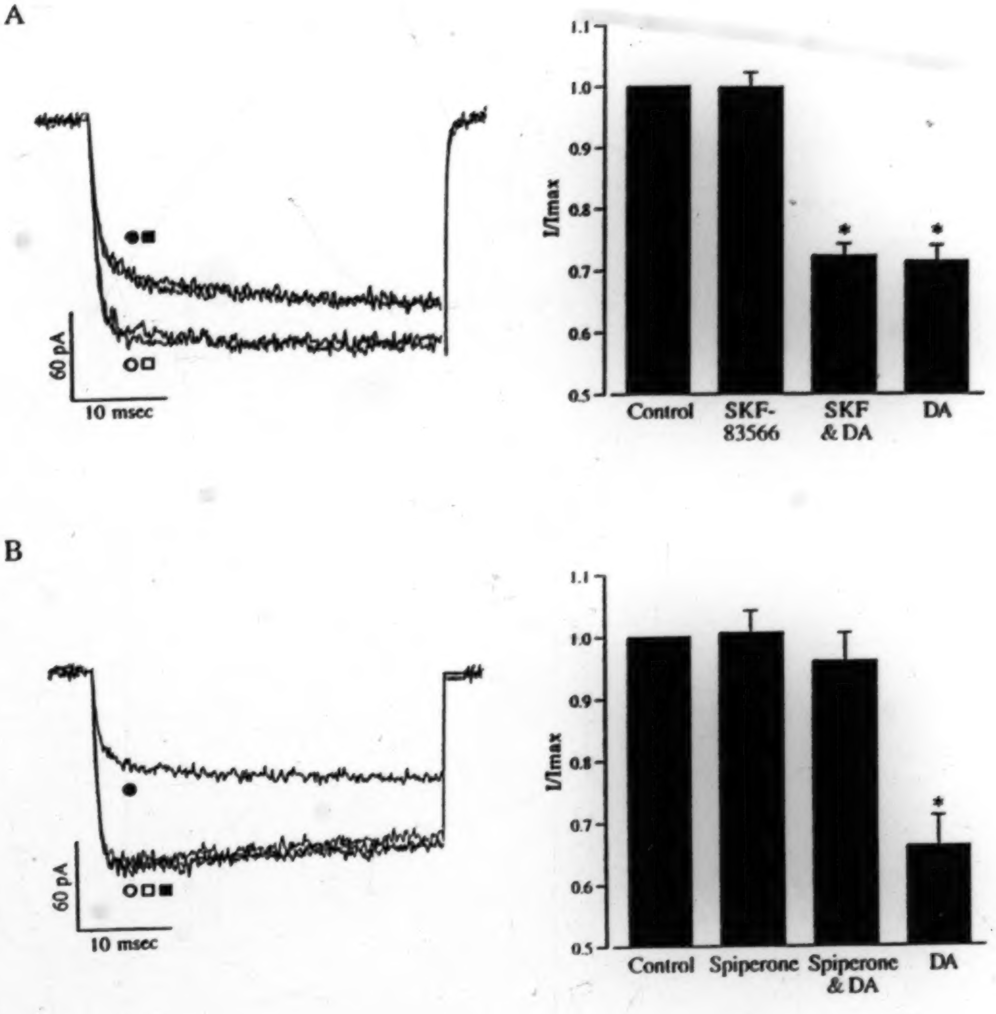


Fig. 5.7. Effects of the dopamine D_1-agonist, SKF 38393, on voltage-dependent Ba^{2+} currents in identified goldfish gonadotrophs. A. Superimposed Ba^{2+} current traces from a representative gonadotroph in the presence (*filled circle*) or absence (*open circle*) of 100 nM SKF 38393. Currents were elicited by a 40-msec voltage-step to +10 mV from a holding potential of -80 mV. B. Current-voltage relationship of the Ba^{2+} current ($n = 7$) evoked by 200-msec voltage-steps to the potentials indicated (holding potential = -80 mV) in the presence (*filled circles*) or absence (*open circles*) of 100 nM SKF 38393. All currents were normalized to the maximal inward current and are presented as mean ± SEM.

Fig. 5.8. Effects of dopamine D_1 and D_2 receptor antagonists on dopamine-induced inhibition of voltage-dependent Ba^{2+} currents in identified goldfish gonadotrophs. A. The D_1 antagonist SKF 83566 failed to block the inhibitory dopamine effect. Left panel: superimposed Ba^{2+} current traces from a representative gonadotroph evoked by a 40-msec voltage step to +10 mV (holding potential = -80 mV) in presence of 1 µM SKF 83566 (*open square*), 10 nM dopamine (*filled circle*), 1 µM SKF 83466 and 10 nM dopamine (*filled square*) or vehicle control (*open circle*). Right panel: Ba^{2+} currents were normalized to the maximal Ba^{2+} current amplitude observed under control conditions and are presented as mean ± SEM (n = 5). B. The D_2 antagonist spiperone blocked the inhibitory dopamine effect. Left panel: Superimposed Ba^{2+} current traces evoked by a 40-msec voltage step to +10 mV (holding potential = -80 mV) in presence of 1 µM spiperone (*open square*), 10 nM dopamine (*filled circle*), 1 µM spiperone and 10 nM dopamine (filled square) or vehicle control (open circle). Right panel: Ba^{2+} currents were normalized to the maximal Ba^{2+} current amplitude observed under control conditions and are presented as mean ± SEM (n = 5). *Asterisk* indicates significant difference compared to control values; $P < 0.05$, ANOVA, followed by Fisher's LSD test.


A
●■
○□
60 pA
10 msec
1.1
1.0
0.9
0.8
0.7
0.6
0.5
I/Imax
*
*
Control
SKF-83566
SKF & DA
DA
B
●
○□■
60 pA
10 msec
1.1
1.0
0.9
0.8
0.7
0.6
0.5
I/Imax
*
Control
Spiperone
Spiperone & DA
DA

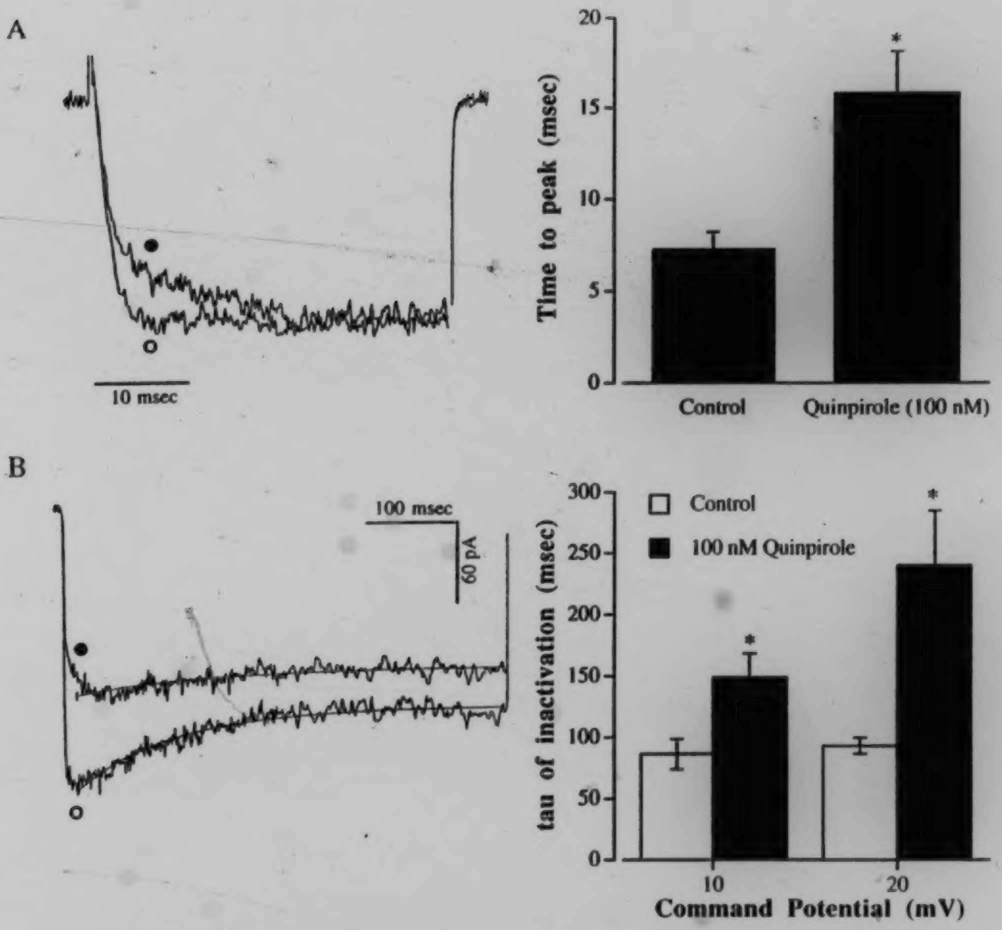


Fig. 5.9. Effects of quinpirole on the activation and inactivation kinetics of Ba^{2+} currents in identified goldfish gonadotrophs. A. Left panel: superimposed Ba^{2+} current traces normalized to their maximal current amplitude. Currents were elicited by a 40-msec voltage step to +10 mV (holding potential = -80 mV) in presence (*filled circle*) or absence (*open circle*) of 100 nM quinpirole. Right panel: pooled results from 12 gonadotrophs showing the time to peak current in the absence or presence of 100 nM quinpirole. B. Left panel: superimposed Ba^{2+} current traces from a representative gonadotroph elicited by a 500-msec voltage step to +10 mV (holding potential = -80 mV) in the presence (*filled circles*) or absence (*open circles*) of 100 nM quinpirole. Solid-lines represents a single exponential fit of the inactivating phase of the voltage-dependent Ba^{2+} current. Right panel: pooled results from 8 gonadotrophs showing the time constant (tau) of inactivation in the presence or absence of 100 nM quinpirole. *Asterisk* indicates significant difference compared to control values; $P < 0.05$, unpaired *t*-test.

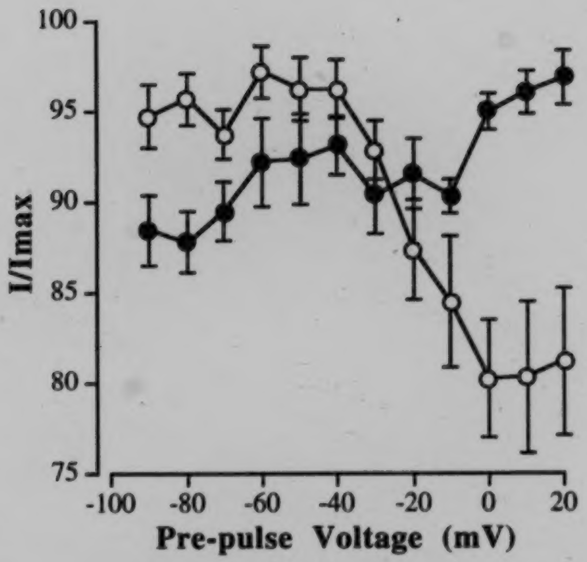
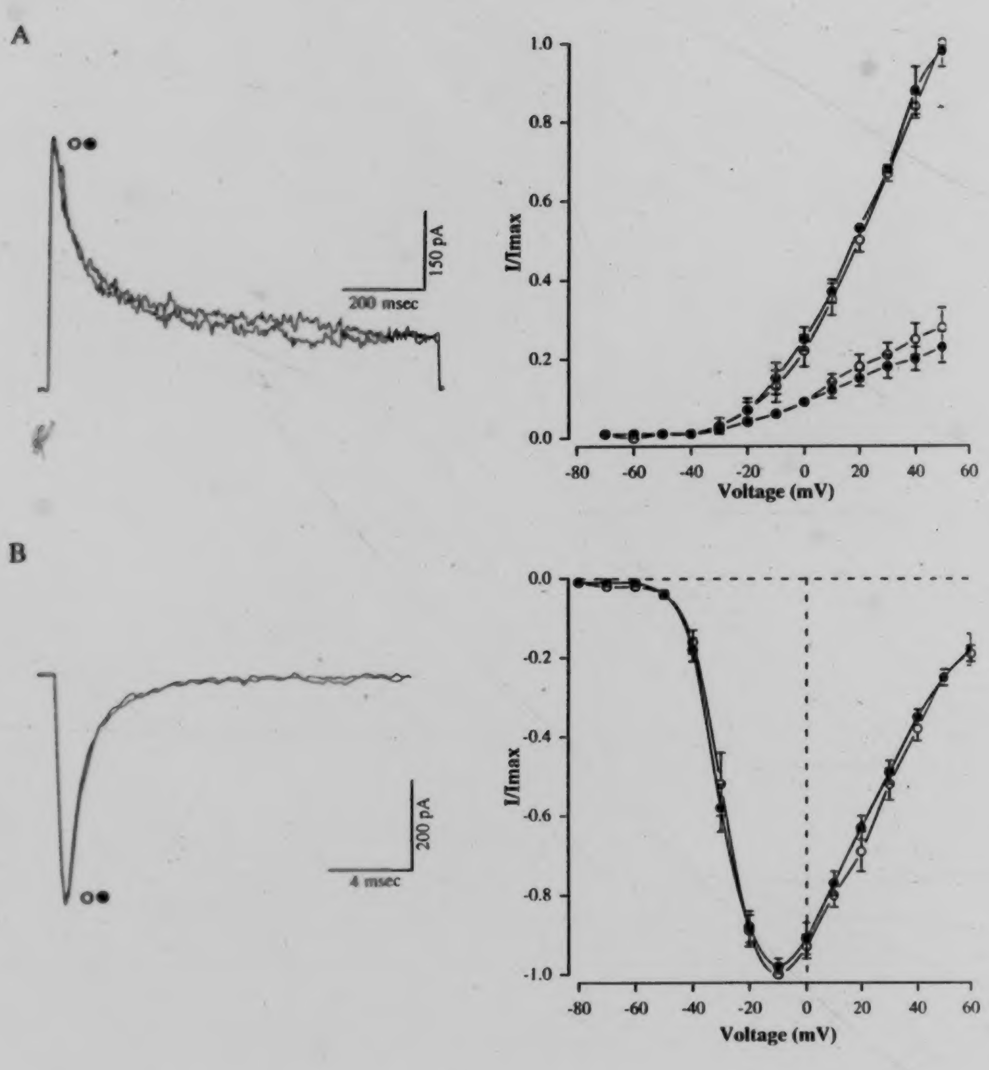


Fig. 5.10. Effects of quinpirole on steady-state inactivation of voltage-dependent Ba^{2+} currents in identified goldfish gonadotrophs. In the absence (*open circles*) or presence of 100 nM quinpirole (*filled circles*), steady-state inactivation curves were generated by stepping the membrane potential to between -90 and +20 mV for 500 msec before stepping to a 20 msec test potential of +10 mV. All currents were normalized to the maximal inward current and are presented as mean ± SEM.

Fig. 5.11. Effects of dopamine on voltage-dependent K+ and Na+ currents in identified goldfish gonadotrophs. A. Perforated patch-clamp recordings of voltage-dependent K+ currents in the presence of dopamine. Left panel: superimposed K+ current traces from a representative gonadotroph evoked by a 1-sec voltage-step to +20 mV from a holding potential of -80 mV before (*open circle*) and during (*filled circle*) the application of 100 nM dopamine. Right panel: current-voltage relationship of the voltage-dependent K+ current in the presence (*filled circles*) or absence (*open circles*) of 100 nM dopamine (*n* = 3). B. Perforated patch-clamp recordings of voltage-dependent Na+ currents in the presence of dopamine. Left panel: superimposed Na+ current traces from a representative gonadotroph evoked by a 40-msec voltage-step to 0 mV from a holding potential of -90 mV before and during the application of 100 nM dopamine. Right panel: current-voltage relationship of the voltage-dependent Na+ current in the presence (*filled circles*) or absence (*open circles*) of 100 nM dopamine (*n* = 5). All currents in A and B were normalized to the maximal inward current under control conditions and are presented as mean ± SEM.


A
○●
150 pA
200 msec
I/Imax
1.0
0.8
0.6
0.4
0.2
0.0
-80
-60
-40
-20
0
20
40
60
Voltage (mV)
B
○●
200 pA
4 msec
I/Imax
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-80
-60
-40
-20
0
20
40
60
Voltage (mV)

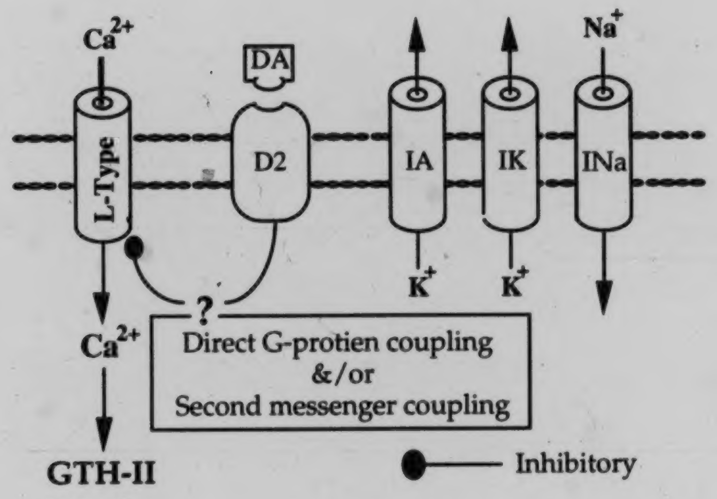


Fig. 5.12. Dopamine actions on voltage-dependent ionic channels in goldfish gonadotrophs. Abbreviations include; dopamine, DA; dopamine D_2 receptor, D2; I_A, fast transient K^+ currents; I_K, delayed rectifier K^+ currents.

Chapter 6

GONADOTROPIN-RELEASING HORMONE-INDUCED INTRACELLULAR CALCIUM SIGNALING IN GOLDFISH GONADOTROPHS

INTRODUCTION

Agonist-induced increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) trigger hormone secretion in many endocrine cell types, including gonadotrophs (reviewed in Stojilkovic and Catt, 1992; Hille *et al.*, 1994; Stojilkovic *et al.*, 1994). In the well characterized rat gonadotroph model, both intracellular and extracellular Ca^{2+} stores contribute to GnRH-induced increases in $[Ca^{2+}]_i$ (Shangold *et al.*, 1988; Stojilkovic and Catt, 1992; Stojilkovic *et al.*, 1994; Hille *et al.*, 1994) and hormone secretion (Stojilkovic *et al.*, 1990, 1992). The intracellular Ca^{2+} response to GnRH is characterized by repetitive transient increases in $[Ca^{2+}]_i$, the frequency of which is modulated by GnRH concentration. These Ca^{2+} oscillations are initiated by the rapid release of intracellular Ca^{2+} from $InsP_3$-sensitive Ca^{2+} stores (Hille *et al.*, 1994; Stojilkovic *et al.*, 1994). The ensuing rise in $[Ca^{2+}]_i$ stimulates exocytosis, as determined by measuring alterations in membrane capacitance as an index of changes in membrane surface area (Tse *et al.*, 1993), and has been linked to the early phase of LH secretion (Stojilkovic *et al.*, 1990). On the other hand, the maintenance of GnRH-induced Ca^{2+} oscillations and LH secretion during prolonged exposure to GnRH is dependent on extracellular Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Stojilkovic and Catt, 1992;

Stojilkovic *et al.*, 1994; Hille *et al.*, 1994). Therefore, intracellular and extracellular Ca^{2+} stores work in a cooperative fashion to tightly regulate $[Ca^{2+}]_i$ and LH secretion in GnRH-stimulated rat gonadotrophs.

Indirect studies have indicated that stimulus-secretion coupling in goldfish gonadotrophs is also mediated by an increase in $[Ca^{2+}]_i$. In particular, the evidence for a role of Ca^{2+} in mediating the actions of the two native GnRHs, sGnRH and cGnRH-II, has been primarily attained by monitoring GTH-II secretion in static incubation and perifusion experiments on dispersed goldfish pituitary cells. Static incubation experiments have been used to monitor the GTH-II response during prolonged (2 hr) exposure to GnRH, while perifusion studies have been used to monitor short-term responses to brief stimuli (5 - 10 min). During both short-term and prolonged exposure to sGnRH, GTH-II secretion is partially reduced by removal of extracellular Ca^{2+} or inhibition of Ca^{2+} influx by inorganic or organic Ca^{2+} channel blockers. In contrast, the GTH-II release response to prolonged exposure to cGnRH-II is abolished by removal of Ca^{2+} influx (Jobin and Chang, 1992a). However, during short-term exposure to cGnRH-II, GTH-II secretion is reduced by inhibition of Ca^{2+} entry (Jobin *et al.*, 1996). In Ca^{2+}-imaging studies on mixed populations of dispersed goldfish pituitary cells, cGnRH-II-, but not sGnRH-stimulated increases in $[Ca^{2+}]_i$ were abolished by the removal of extracellular Ca^{2+} (Jobin and Chang, 1992b). These findings indicated that extracellular Ca^{2+} influx participates in the actions of both native GnRHs, whereas mobilization of intracellular Ca^{2+} may only be involved during sGnRH action. In support of this view, only sGnRH stimulates the production of $InsP_3$ (Chang *et al.*, 1995), which plays a prominent role in the mobilization of intracellular Ca^{2+} in many cell types, including rat, avian and bovine gonadotrophs (Davidson *et al.*, 1990; Stojilkovic *et al.*, 1994; Hille *et al.*, 1994).

In this chapter, I tested the hypothesis that GnRH-stimulates an increase in $[Ca^{2+}]_i$ in goldfish gonadotrophs and examined the involvement of intracellular Ca^{2+} release and Ca^{2+} influx in mediating the response to sGnRH and cGnRH-II. To monitor more directly changes in $[Ca^{2+}]_i$ during GnRH-receptor activation, single-cell Ca^{2+}-imaging experiments were conducted on morphologically identified goldfish gonadotrophs. The membrane permeant intracellular Ca^{2+} indicator, fura-II/acetoxymethyl ester (AM; Grynkiewich *et al.*, 1985; Tsien *et al.*, 1985), was used in combination with dual excitation wavelength epifluorecent microscopy to monitor temporal and spatial changes in $[Ca^{2+}]_i$. To achieve high spatial resolution, changes in $[Ca^{2+}]_i$ were monitored using a X100 oil-immersion objective. Perforated-patch-clamp recording techniques were also used to investigate the actions of the two native GnRHs on voltage-dependent Ca^{2+} channels. In general, acute application (≤ 2-min) of sGnRH or cGnRH-II stimulated an increase in $[Ca^{2+}]_i$, which was partially independent of extracellular Ca^{2+} influx. Consistent with these observations, neither GnRHs affected the amplitude of voltage-dependent Ca^{2+} currents.

MATERIALS AND METHODS

General

Common goldfish (8-13 cm in body length) were purchased from Grassyforks Fisheries (Martinsville, IN) or Ozark Fisheries (Stoutland, MO) and maintained as previously described (Chapter 2). Sexually regressed and pre-spawning male and female goldfish were used, unless otherwise indicated. Fish were anesthetized in 0.05% tricaine methanesulfonate prior to decapitation. Pituitary cells were dispersed using a controlled trypsin/DNAse treatment procedure modified from Chang *et al* (1990a; Chapter 5). The cells were subsequently resuspended in plating medium supplemented with 10% horse serum and cultured overnight under 5% CO_2, saturated humidity, and at 28 °C. For electrophysiological and single-cell Ca^{2+}-imaging studies, dispersed pituitary cells were cultured for 16 hr in poly-L-lysine (0.01 mg/ml) coated glass-bottom petri dishes. Goldfish gonadotrophs were identified on the basis of their unique morphological characteristics as previously described (Chapter 2).

Measurement of $[Ca^{2+}]_i$

Fura-II/AM loading. The $[Ca^{2+}]_i$ in single identified goldfish gonadotrophs was determined using the fluorescent Ca^{2+} indicator, fura-II (Tsien *et al.*, 1985). Dispersed goldfish pituitary cells were rinsed once with testing medium and then incubated for 60 min at 28 °C with 10 µM of the membrane permeant form of fura-II, fura-II/acetoxymethyl ester (AM) and 25 µg/ml pluronic F-127 (Molecular Probes, Eugene, OR). Fura-II/AM was dissolved in DMSO with 25 mg/ml pluronic F-127 to obtain a 10 mM stock solution, which was subsequently diluted to its final concentration in testing media immediately prior to use. The addition of the non-ionic dispersing agent, pluronic F-127, helped to solubilize the fura-II/AM, resulting in a more uniform dye distribution (Poenie *et al.*, 1986). The final DMSO concentration was 0.1%, which does not affect ionic currents or GTH-II secretion in goldfish. The concentration of fura-II/AM, DMSO, pluronic F-127, as well as incubation time and temperature, were manipulated to result in optimal loading of identified goldfish gonadotrophs with fura-II/AM (Fig. 6.1). Similar fura-II/AM loading conditions have been used with African catfish gonadotrophs (Rebers *et al.*, 1995). Following fura-II/AM loading, the dispersed pituitary cells were rinsed, twice, with modified testing medium (Fura-II medium: modified M-199 with Hank's salts, 0.385 g/l $NaHCO_3$, 25 mM HEPES, 100 000 U/l penicillin, 100 mg/l streptomycin, 0.1% BSA, prepared with the addition of 136 mM NaCl, 5 mM $CaCl_2$ and 5.4 mM KCl, but without the addition of phenol red; pH adjusted to 7.2 with NaOH; courtesy of Dr. S.S. Stojilkovic, ERRB, NICHD, NIH, Bethesda, MD) to remove excess fura-II/AM. Intracellular Ca^{2+} imaging was performed within 5 to 60 min of fura-II/AM loading at 18 °C to 20 °C. The bath contained < 200 µl of fura-II testing medium and was continuously perfused at a rate of 2 ml/min using a gravity-driven superfusion system. The outflow was placed near the cell, resulting in complete solution exchange around the cell within 2 sec. For experiments using high extracellular K^+ concentrations, 25 mM NaCl was replaced with equimolar KCl, resulting in an extracellular KCl concentration of ≈30 mM.

Determination of relative changes in $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ were made by determining the ratio of the emission intensity of fura-II fluorescence subsequent to excitation at two different wave lengths as previously described (Tsien *et al.*, 1985). Single fura-II/AM-loaded gonadotrophs were imaged under epifluorescence microscopy using a Ziess Axiovert 135 inverted microscope with a Plan-Neofluar 100 X/1.3 NA oil-immersion objective (Ziess, Ontario, Canada). Fura-II fluorescence was excited by UV light from a 100 watt Hg/Xe-arc lamp, which was alternately passed through 340 nm and 380 nm excitation filters using a computer controlled rotating filter wheel. To reduce photobleaching and photodamage during recording, exposure times were minimized and the excitation light intensity was regulated by placing neutral density filters in the excitation light path. The excitation light was reflected toward the image field by a 430 nm dichroic mirror. Emitted light was collected at 510 nm and detected by an intensified charged coupled device (CCD) camera (Paultek Imaging, Nevada City, CA). Ratio images (340/380) were digitized and stored using a Macintosh Quadra 950 computer equipped with a Quickcapture frame grabber board (Data Translation, Ontario, Canada). Ratioed images were acquired every 5 sec to 10 sec using the image capture program Ratio 1.3 (kindly provided by Dr. S. B. Kater, University of Utah School of Medicine).

Estimation of $[Ca^{2+}]_i$ and Data Analyses. Changes in $[Ca^{2+}]_i$ are presented as estimated $[Ca^{2+}]_i$ or as a ratio of the fluorescent emission intensity following alternate excitation at 340 nm and 380 nm (340/380 ratio). The $[Ca^{2+}]_i$ was estimated using the following relation developed by Grynkiewich *et al* (1985):

$$[Ca^{2+}] = K_d Q \frac{(R - R_{\min})}{(R_{\max} - R)} \quad [1]$$

where: R represents the fluorescence emission intensity ratio F_{340}/F_{380} at the point of interest; K_d is the apparent Ca^{2+} dissociation constant for fura-II and Q is the ratio of fluorescence emission intensities, F_{min}/F_{max}, measured at 380 nm excitation in the absence of Ca^{2+} (F_{min}) and in the presence of saturating Ca^{2+} concentrations (F_{max}). Ratios corresponding to the minimum (R_{min}) and maximum (R_{max}) fluorescence were obtained in zero and saturating Ca^{2+} concentrations, respectively. The K_d of fura-II and the relation, Fmin/Fmax, were determined at 18 °C to 20 °C by measuring the fluorescence signals following alternate excitation at 340 nm and 380 nm of fura-II pentapotassium salt (100 µM) in cell-free solutions of known Ca^{2+} concentration entrapped in glass capillary tubes with a path length of 20 µm (Vitro Dynamics, Rockaway, NJ). Calibration solutions of known Ca^{2+} concentrations (zero to 39.8 µM free Ca^{2+}) were prepared using a Ca^{2+} Calibration Buffer Kit with magnesium II (Molecular Probes, Eugene, OR). The ionic composition of the calibration solutions included: zero to 10 mM CaEGTA, 1 mM free Mg^{2+}, 100 mM KCl and 10 mM MOPS (pH = 7.2). To measure $[Ca^{2+}]_i$, images acquired at 340 nm and 380 nm were ratioed on a pixel-by-pixel basis and $[Ca^{2+}]_i$ was estimated according to equation 1 using the values determined as described above: K_d = 265.00 nM, Q = 1.95, R_{min} = 0.78 and R_{max} = 3.72.

For the determination of relative changes in $[Ca^{2+}]_i$, mean numerical values of intensities (scale of 0 to 256) obtained at 340 nm and 380 nm excitation were determined from a rectangular region that occupied the majority of the cell area and did not extend beyond the cell margin. The raw data recorded in response to excitation at 340 nm and 380 nm were corrected for the background signal recorded in the absence of fluorescent dye as described in Silver *et al* (1992). Briefly, the background intensity recorded at 340 nm and 380 nm from regions of the dish which did not contain any cells were subtracted from the fluorescent intensities before obtaining the ratio value. This background correction accounted for incomplete block of excitation light, the fluorescence of optical components, including the coverslip and extracellular medium and the weak halo of reflected fluorescence around the cells. In unloaded goldfish gonadotrophs, cell autofluorescence was negligible. Due to variation in basal 340/380 ratio values ($[Ca^{2+}]_i$) between experiments, the data were normalized to the mean value obtained in the first three ratio images and were expressed as % pretreatment. All values in the text and figures are reported as mean ± SEM. Differences between groups were considered to be significant when $P < 0.05$.

Electrophysiological Recordings

Whole-cell, voltage-clamp recordings (Hamill *et al.*, 1981) were performed at room temperature (18 °C to 20 °C) using nystatin-perforated patch recording techniques (Korn and Horn, 1989) as described in chapter 3. Briefly, after obtaining electrical access to the cell ($R_s = 26.3 \pm 1.8$ MΩ; $n = 10$), currents were recorded in voltage-clamp mode using a Dagan 3900 integrating patch-clamp amplifier. Series resistance compensation was optimized and usually ranged between 10 and 18 mΩ. Current records were corrected for linear leakage and capacitance using a P/-4 procedure (Bezanilla and Armstrong, 1977). Pulse generation, and data acquisition were carried out using an AT 486 2DX compatible computer equipped with a digidata 1200 interface in conjunction with pCLAMP programs (Axon Instruments, Foster City, CA). The pipette solution contained (in mM): 70 Cs-glutamate, 70 CsCl, 2 MgCl2, 10 HEPES and 160 µg/ml Nystatin (pH adjusted to 7.2 with TrisCl). The composition (in mM) of the external solution for recordings of voltage-dependent Ba^{2+} currents (through voltage-dependent Ca^{2+} channels) was (in mM): 130 N-methyl-D-glucamine-Cl, 20 $BaCl_2$, 1.0 $MgCl_2$, 2.5 KCl, 8 glucose, 10 HEPES (pH adjusted to 7.2 with HCl). The effects of all drug applications on Ca^{2+} currents were continuously monitored by giving a 40 msec voltage-step to 0 mV from a holding potential of -70 mV, every 5 sec. Alternatively, current-voltage relationships were generated using 200 msec voltage-ramps from -80 mV to +80 mV, every 5 sec. Data analysis was performed using an AT 486 2DX compatible computer in conjunction with Clampfit (Axon Instruments, Foster City, CA). The junction potential for Ba^{2+} current recordings was determined to be less than 3.0 mV using the JPCalc program (Barry, 1994) and were not corrected for. All values in the text are reported as mean ± SEM. Differences between groups were considered to be significant when $P < 0.05$ using paired t test.

Drugs

To make stock solutions, sGnRH and cGnRH-II (Peninsula Laboratories, Belmont, CA) were dissolved in distilled de-ionized water. GnRH antagonist E ([Ac-Δ^3-Pro^1, 4F-D-Phe^2, D-Trp^{3,6}]mGnRH; analog E of Murthy *et al.*, 1993), kindly provided by Dr. R. E. Peter at the University of Alberta (Edmonton, Alberta), was dissolved in a primary solvent containing propylene glycol and fura-II testing medium at a ratio of 60:40 (v:v) and subsequently diluted to the final concentration in fura-II testing medium. Nifedipine and S(-)-Bay K 8644 (Research Biochemicals Incorporated, Natick, MA), as well as ionomycin (Calbiochem, San Diego, CA) were dissolved in ethanol. Aliquots of stock solutions were stored at -20 °C until use, when they were diluted to final concentrations in fura-II testing medium for fura-II studies or saline for electrophysiological studies. The highest concentration of ethanol or DMSO was 0.1%, which had no effect on either basal hormone release or ionic currents.

RESULTS

Measurement of $[Ca^{2+}]_i$ in goldfish gonadotrophs

Morphologically identified goldfish gonadotrophs, bathed in Fura-II medium with 5 mM extracellular Ca^{2+}, maintained a wide range of estimated resting $[Ca^{2+}]_i$ with a mean value of 70.3 ± 4.6 nM (mean ± SEM, $n = 76$; Fig. 6.2). Resting $[Ca^{2+}]_i$ was stable during 5-min recording periods in which gonadotrophs were alternately excited at 340 nm and 380 nm every 10 sec (data not shown). The ability of goldfish gonadotrophs to maintain low levels of resting $[Ca^{2+}]_i$ against the electrochemical gradient for Ca^{2+} indicates that their plasma membranes are intact.

In the present study, the Ca^{2+} ionophore, ionomycin, was used to determine if increases in $[Ca^{2+}]_i$ can be observed under the present fura-II/AM loading conditions. The fluorescent excitation spectra of fura-II shifts to shorter wavelengths as Ca^{2+} concentration increases (Grynkiewicz *et al.*, 1985). Therefore, ionomycin should increase the fluorescent intensity measured following excitation at 340 nm, while the fluorescent intensity measured following excitation at 380 nm should decrease. Thus, a rise in $[Ca^{2+}]_i$ in response to ionomycin should increase the 340/380 ratio. Application of 1 μM ionomycin produced these expected changes in fluorescence (Fig. 6.3), with an estimated net increase in peak $[Ca^{2+}]_i$ of 1125.0 ± 197.8 nM (mean ± SEM; range 742 to 1402 nM, $n = 3$). These results are consistent with known changes in the fura-II excitation spectra in response to an increase in Ca^{2+} concentration (Grynkiewicz *et al.*, 1985), suggesting that the fura-II/AM loading and measurement procedures used in this study result in accurate estimations of $[Ca^{2+}]_i$.

Voltage-dependent Ca^{2+} channel-stimulated increases in $[Ca^{2+}]_i$

Dihydropyridine-sensitive, voltage-dependent Ca^{2+} (Ba^{2+}) currents have been observed in identified goldfish gonadotrophs (Chapter 3) and are believed to participate in GnRH-stimulated GTH-II secretion from mixed populations of dispersed goldfish pituitary cells (Chang *et al.*, 1996). In the present study, the effects of voltage-dependent Ca^{2+} channel activation on $[Ca^{2+}]_i$ were examined using the dihydropyridine-sensitive Ca^{2+} channel activator, Bay K 8644, and high concentrations of extracellular KCl. Application of 10 μM Bay K 8644, which increases voltage-dependent Ba^{2+} currents in identified goldfish gonadotrophs (Chapter 3), elicited a rapid and persistent increase in $[Ca^{2+}]_i$ (Fig. 6.4; $n = 4$). The estimated net increase in peak $[Ca^{2+}]_i$ was 316.3 ± 89.0 nM (range = 67 nM to 653 nM, n = 4). Similarly, application of 30 mM KCl increased $[Ca^{2+}]_i$ by 62.5 ± 32.2 nM (range = 14 nM to 152 nM, n = 4; Fig. 6.5).

Ca^{2+} release from intracellular Ca^{2+} stores

Release of Ca^{2+} into the cytosol from endoplasmic reticulum stores may also contribute to agonist-induced GTH-II secretion. To demonstrate the presence of functional intracellular Ca^{2+} stores in goldfish gonadotrophs, reuptake of cytosolic free Ca^{2+} by the endoplasmic reticulum was blocked by the addition of thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-adenosine 5'-triphosphatase (Ca^{2+}-ATPase). Thapsigargin (1 μM) induced a relatively prompt (within 20 sec) rise in $[Ca^{2+}]_i$ which slowly increased until reaching a stable level after approximately 2 min of incubation (Fig. 6.6). The average maximal increase in estimated $[Ca^{2+}]_i$ in response to thapsigargin was 47.0 ± 19.3 nM (range = 21 nM to 103 nM, n = 4).

GnRH-induced increases in $[Ca^{2+}]_i$

In response to a 1 or 2 min application of either 100 nM sGnRH or 100 nM cGnRH-II, an increase in $[Ca^{2+}]_i$ was observed in 94% (n = 36) of the cells identified as gonadotrophs by their distinct cellular morphologies (Fig. 6.7). A 2 min application of 100 nM sGnRH stimulated a peak increase in estimated $[Ca^{2+}]_i$ of 281.0 ± 44.7 nM (range = 146 nM to 532 nM, n = 8), whereas cGnRH-II stimulated a peak increase in estimated $[Ca^{2+}]_i$ of 288.6 ± 42.7 nM (range = 62 nM to 648 nM, n = 15). An increase in $[Ca^{2+}]_i$ in response to GnRH was also observed in morphologically identified somatotrophs (data not shown). In dispersed goldfish pituitary cells which do not exhibit gonadotroph- or somatotroph-like cellular morphologies (Chapter 2), neither sGnRH nor cGnRH-II stimulated an increase in $[Ca^{2+}]_i$ (Fig. 6.8). These results suggest that GnRH-induced increases in $[Ca^{2+}]i$ act specifically on gonadotrophs and somatotrophs.

The temporal pattern of the $[Ca^{2+}]_i$ response profile after the application of sGnRH and cGnRH-II were dose dependent and reversible (Fig. 6.9). In all gonadotrophs tested, a 2-min application of 100 nM cGnRH-II (n = 15) induced a biphasic increase in $[Ca^{2+}]_i$, consisting of an initial spike phase followed by a sustained, lower amplitude plateau phase (Fig. 6.7 and 6.9). In 6 gonadotrophs tested, application of 10 nM cGnRH-II elicited a biphasic increase in $[Ca^{2+}]_i$ in 5 cells, while 1 cell exhibited a slow-monophasic increase (Fig. 6.9 A). Application of 1 nM cGnRH-II induced slow-monophasic increases in $[Ca^{2+}]_i$ in all cells tested (n = 3; Fig. 6.9 A). Similar response profiles were observed after application of 100 nM (n = 8), 10 nM (n = 4), and 1 nM (n = 4) sGnRH (Fig. 6.7 and 6.9). The onset of both sGnRH- and cGnRH-II-induced increases in $[Ca^{2+}]_i$ became progressively shorter with higher concentrations of GnRH (Fig. 6.9 A and B). Under the current recording conditions and temporal resolution, possible evidence for GnRH-induced oscillatory Ca^{2+} responses were observed in only two cells (Fig. 6.13). Better temporal resolution will be required to more clearly elucidate the temporal patterns of Ca^{2+} signaling in these cells.

Several studies have suggested that both sGnRH and cGnRH-II bind to the same class of GnRH-receptors to stimulate GTH-II secretion. In initial experiments, sequential application of 10 nM sGnRH and 10 nM cGnRH-II increased the estimated $[Ca^{2+}]_i$ by 39 nM and 51 nM, respectively, in the same gonadotroph. In addition, only gonadotrophs which exhibited the same morphological characteristics were used in the present study. These results indicated that, within the same subpopulation of gonadotrophs used in this study, both native GnRHs act on the same cell to stimulate an increase in $[Ca^{2+}]_i$.

Due to the heterogeneity of the $[Ca^{2+}]_i$ response profiles and differences in the latency of the response to various concentrations of sGnRH or cGnRH-II, a dose-response relationship was determined from the net change in peak (or maximal) $[Ca^{2+}]_i$ during a 2-min application of GnRH. To avoid the possible desensitization of the intracellular Ca^{2+} response due to successive applications of GnRH (McArdle *et al.*, 1995), GnRH was generally applied only once per culture dish. Application of 0.001 nM to 100 nM sGnRH or cGnRH-II induced a dose-dependent increase in peak $[Ca^{2+}]_i$ (Fig. 6.10). With the exception of 0.001 nM sGnRH *vs.* 0.001 nM cGnRH-II, no significant differences were observed between sGnRH and cGnRH-II with respect to the net change in peak $[Ca^{2+}]_i$ ($P > 0.05$, unpaired *t*-test, $n = 3$ to 15).

Suppression of GnRH-induced Ca^{2+} responses by a GnRH-receptor antagonist

Hormone release studies have demonstrated that the GnRH analog [Ac-Δ^3-Pro^1, 4F-D-Phe^2, D-Trp^{3,6}]mGnRH (analog E) acts as a 'true' GnRH antagonist in goldfish, as it competitively inhibits GnRH receptor binding without stimulating GTH-II secretion (Murthy *et al.*, 1993). To investigate the effects of analog E (antagonist E) on GnRH-induced increases in $[Ca^{2+}]_i$, 2 µM antagonist E was applied 90 sec before and during the application of a 60-sec pulse of 10 nM sGnRH. The antagonist was then removed and after a 5-min wash period, sGnRH was re-applied in the absence of antagonist E. Application of 2 µM antagonist E abolished the $[Ca^{2+}]_i$ response to 10 nM sGnRH (Fig. 6.11 A, B). There was no difference in the Ca^{2+} response between the antagonist alone or sGnRH in the presence of the antagonist ($P > 0.05$, $n = 6$; Figure 6.11 B). Additionally, the vehicle solution did not alter $[Ca^{2+}]_i$ (net change in peak 340/380 ratio above basal values was 3.0 ± 1.3 %, which is not significantly different than 0 change, $P > 0.05$; one group *t*-test; $n = 6$). However, antagonist E alone did induce a relatively small increase in $[Ca^{2+}]_i$ at the dose used in the present study ($P < 0.05$, $n = 6$; Figure 6.11 A, B).

Evidence for localized increases in $[Ca^{2+}]_i$

In many cell types, activation of Ca^{2+} mobilizing receptors stimulates spatially localized increases in $[Ca^{2+}]_i$. In the present study, GnRH induced both localized and non-localized increases in $[Ca^{2+}]_i$. A localized increase was observed in 76.5% ($n = 17$) of the cells in response to a 2-min application of 100 nM cGnRH-II (Fig. 6.12). In these cells, localized increases in $[Ca^{2+}]_i$ appeared near to the plasma membrane and progressively decreased towards the center of the cell. In addition, the localized increase in $[Ca^{2+}]_i$ was observed toward one pole of the cell. To be consistent with previous reports (Rawlings *et al.*, 1991), the localized increases in $[Ca^{2+}]_i$ observed in these cells will be referred to as polarized Ca^{2+} responses. Polarized Ca^{2+} responses were also observed in 62.5 % ($n = 8$) of the gonadotrophs following a 2-min application of 100 nM sGnRH. Non-polarized $[Ca^{2+}]_i$ responses were also observed in response to 100 nM sGnRH (Figure 6.13) or 100 nM cGnRH-II. These non-polarized Ca^{2+} responses are characterized by an increase in $[Ca^{2+}]_i$ throughout the cytoplasm with numerous small Ca^{2+} "hot spots" dispersed throughout.

Release of intracellular Ca^{2+} from thapsigargin-sensitive endoplasmic reticulum stores also occurs in a localized region of the cell and appears to be greatest nearer to the plasma membrane (Fig. 6.14). These results suggest that release of Ca^{2+} from intracellular stores is also localized and has a similar spatial pattern as that induced by GnRH. However, whether thapsigargin- and GnRH-sensitive pools are similar remains to be determined.

Involvement of extracellular Ca^{2+} during GnRH action

The possible involvement of extracellular Ca^{2+} influx in mediating GnRH action was investigated by removing extracellular Ca^{2+} from the medium or by the addition of inorganic voltage-dependent Ca^{2+} channel blockers. Addition of 50 µM $CdCl_2$ decreased the peak $[Ca^{2+}]_i$ response to 100 nM cGnRH-II compared to control values (Fig. 6.15; $P < 0.05$, ANOVA-repeated measures followed by paired *t*-test, $n = 6$). However, the cGnRH-II-stimulated increase in peak $[Ca^{2+}]_i$ observed in the presence of Cd^{2+} was not significantly different from responses obtained following removal of Cd^{2+} (washout; $P > 0.05$, ANOVA-repeated measures followed by paired *t*-test). In light of the fact that the inhibitory effects of Cd^{2+} on voltage-dependent Ca^{2+} currents are completely reversible (Chapter 3), it is unclear if blockade of Ca^{2+} entry affected the $[Ca^{2+}]_i$ response to cGnRH-II. In addition, it is possible that multiple exposure to GnRH desensitized the intracellular Ca^{2+} response to subsequent applications. To minimize potential desensitization of sequential responses caused by repeated GnRH application, the initial control GnRH pulse was eliminated in subsequent experiments designed to investigate the involvement of Ca^{2+}. In addition, the wash period between the termination of treatments altering extracellular Ca^{2+} availability and application of the washout control pulse was extended from 4.5 to 7 min. Exposure to Ca^{2+}-free medium for 2 min prior to the application of a 2-min pulse of 100 nM cGnRH-II did not affect the peak $[Ca^{2+}]_i$ response or the temporal profile of the Ca^{2+} response compared to washout controls (Fig. 6.16; $P > 0.05$, paired *t*-test, n = 3). Similarly, the $[Ca^{2+}]_i$ response to 100 nM sGnRH was not affected by the addition of 2 mM $CoCl_2$ (Fig. 6.17; $P > 0.05$, paired *t*-test, $n = 6$). Since the potential of desensitization cannot be entirely eliminated even in these latter experiments, it remains unclear whether extracellular Ca^{2+} entry plays a role in the acute $[Ca^{2+}]_i$ response to the two native GnRHs. Nevertheless, the persistence of an $[Ca^{2+}]_i$ response in the presence of $CdCl_2$, $CoCl_2$ and Ca^{2+} solutions strongly suggests that mobilization of Ca^{2+} from intracellular stores participates in the acute actions of sGnRH and cGnRH-II on goldfish gonadotrophs.

Although the involvement of extracellular Ca^{2+} influx in acute (≤ 2-min) actions of GnRH cannot be firmly established in single-cell fura-II imaging experiments, there is evidence that the intracellular Ca^{2+} stores and extracellular Ca^{2+} entry through voltage-dependent Ca^{2+} channels are closely linked and may be interrelated. Like GnRH, activation of voltage-dependent Ca^{2+} channels by either Bay K 8644 or 30 mM KCl stimulates localized increases in $[Ca^{2+}]_i$ (Fig. 6.18). During successive application of 2-min pulses of 100 nM cGnRH-II followed by 10 µM Bay K 8644, polarized Ca^{2+} responses were observed in the same discreet area of the cell (Fig. 6.18 A, B). Interestingly, application of 10 µM nifedipine, which reduces voltage-dependent Ba^{2+} currents in these cells (Chapter 3), stimulated a polarized increase in $[Ca^{2+}]_i$; however, the magnitude of nifedipine-induced increases in $[Ca^{2+}]_i$ varied widely from cell to cell (Fig. 6.19). These results suggest that a decrease in Ca^{2+} entry stimulates a rapid release of Ca^{2+} from intracellular Ca^{2+} stores. As with Bay K 8644, successive application of nifedipine and cGnRH-II induced polarized Ca^{2+} responses in the same distinct region of the cell (Fig. 6.19). Taken together, it is likely that both GnRH and nifedipine treatment activate the same intracellular Ca^{2+} pools.

Possible actions of GnRH on voltage-dependent Ba^{2+} currents

To further investigate the possible link between GnRH receptor activation and voltage-dependent Ca^{2+} channels, perforated-patch voltage-clamp recordings of isolated Ba^{2+} currents were performed. As in previous experiments, Ba^{2+} was used as the charge carrier through voltage-dependent Ca^{2+} channels for the reasons outlined in Chapter 3. Application of 100 nM cGnRH-II or 100 nM sGnRH did not alter the amplitude or activation time course of voltage-dependent Ba^{2+} currents in identified goldfish gonadotrophs (Table 6.1; Fig. 6.20 A and B). As GnRH induced a rapid biphasic increase in $[Ca^{2+}]_i$, which may involve Ca^{2+} entry through voltage-dependent Ca^{2+} channels, current-voltage relationships were generated using voltage-ramps rather than a series of successive voltage-steps. This allows for the determination of the current-voltage relationship of the voltage-dependent Ba^{2+} current within 200 msec compared to several seconds. A 200 msec voltage-ramp from -80 mV to +80 mV, elicited an inward Ba^{2+} current at membrane potentials more depolarized than -30 mV, which reached a maximum amplitude near +16 mV (Fig. 6.20). These values are similar to those obtained using a series of successive voltage-steps to generate a current-voltage curve under similar recording conditions (Chapter 5). Application of either sGnRH or cGnRH-II did not alter the activation threshold, current-voltage relationship or apparent reversal potential of the Ba^{2+} currents in identified goldfish gonadotrophs (Fig. 6.20 A and B). These results indicate that acute activation of GnRH receptors does not affect voltage-dependent Ca^{2+} channels in goldfish gonadotrophs.

DISCUSSION

Regulation of $[Ca^{2+}]i$ in unstimulated goldfish gonadotrophs

The $[Ca^{2+}]_i$ in unstimulated secretory cells is tightly regulated due to its stimulatory role in excitation-secretion coupling. Accordingly, in unstimulated goldfish gonadotrophs, low resting $[Ca^{2+}]_i$ are maintained in the presence of high concentrations of extracellular Ca^{2+}, indicating that Ca^{2+} homeostatic mechanisms are operating in these cells. This is further demonstrated by the rapid return of $[Ca^{2+}]_i$ to base-line levels following the termination of ionomycin treatment. Although some of the Ca^{2+} may be extruded from the cell by plasma membrane Ca^{2+} pumps, cytosolic Ca^{2+} is likely re-sequestered due to the activity of a thapsigargin-sensitive endoplasmic reticulum Ca^{2+}-ATPase. The presence of this Ca^{2+}-ATPase in goldfish gonadotrophs is demonstrated in this study. However, unlike the situation in rat gonadotrophs (Stojilkovic *et al.*, 1993), there is no evidence of thapsigargin-induced Ca^{2+} oscillations in goldfish gonadotrophs under the current experimental conditions. In many cells types, thapsigargin-sensitive, intracellular Ca^{2+} stores contribute to $InsP_3$-induced Ca^{2+} release during the activation of Ca^{2+}-mobilizing receptors (Berridge 1993), including GnRH-receptors in rat gonadotrophs (Stojilkovic *et al.*, 1994). Whether the thapsigargin-sensitive stores in goldfish gonadotrophs are sensitive to $InsP_3$ and/or other second messengers remains to be determined.

In many pituitary cell types, spontaneous action potential activity promotes Ca^{2+} entry through voltage-dependent Ca^{2+} channels. In the present study, Bay K 8644 and high concentrations of extracellular KCl stimulate an increase in $[Ca^{2+}]_i$ in goldfish gonadotrophs. These findings are consistent with the presence of L-type Ca^{2+} channels in goldfish gonadotrophs, as previously observed using whole-cell, patch-clamp recording techniques (Chapter 3). In addition, as Bay K 8644 increases Ca^{2+} current by maintaining Ca^{2+} channels in the open state rather than opening closed channels (Kokubin and Reuter, 1984), these data suggest that in unstimulated goldfish gonadotrophs, at least some of the Ca^{2+} channels are in the open state. In this regard, spontaneous Na^+- and Ca^{2+}- dependent action-potential activity has been observed in these cells (Chapter 3). Thus, it appears that there is a continuous influx of Ca^{2+} through voltage-dependent Ca^{2+} channels.

The entry of Ca^{2+} during spontaneous action potential activity in unstimulated gonadotrophs may be important for maintaining intracellular Ca^{2+} stores. In the present study, nifedipine, which reduces voltage-dependent Ca^{2+} currents in these cells (Chapter 3), stimulates an increase in $[Ca^{2+}]_i$. Additionally, in other studies (Jobin, 1993), inhibition of Ca^{2+} entry by the application of nifedipine or inorganic Ca^{2+} channel blockers, as well as the removal of extracellular Ca^{2+}, elicits a transient increase in GTH-II secretion followed by a sustained decrease in basal secretion (Jobin, 1993). These results suggest that a decrease in Ca^{2+} entry or the corresponding decrease in $[Ca^{2+}]_i$ either stimulates the release of Ca^{2+} from, or inhibits Ca^{2+} sequestration by, intracellular Ca^{2+} stores. On the other hand, if cells are pre-exposed to medium containing high concentrations of Ca^{2+}, the GTH-II release response to subsequent removal of extracellular Ca^{2+} was augmented compared to the response following treatment with normal Ca^{2+} concentrations (Jobin, 1993). These results suggest that an elevated Ca^{2+} entry increases the concentration of Ca^{2+} within the intracellular Ca^{2+} stores (Jobin 1993). Taken together, these results suggest that the activity of the voltage-dependent Ca^{2+} channels and the resulting changes in $[Ca^{2+}]_i$ influence the concentration of Ca^{2+} within the intracellular Ca^{2+} stores. Whether the intracellular Ca^{2+} release sites are directly linked to voltage-dependent Ca^{2+} channels or indirectly linked to them through second messengers remains to be determined. However, preliminary studies indicate that the intracellular Ca^{2+} release sites and voltage-dependent Ca^{2+} channels are located in the same region of the cell.

GnRH receptor activation stimulates an increase in $[Ca^{2+}]i$

An increase in $[Ca^{2+}]_i$ is believed to be important in regulating excitation-secretion coupling in goldfish gonadotrophs (Jobin and Chang, 1992a; Jobin *et al.*, 1996). Consistent with this view, sGnRH and cGnRH-II stimulate an increase in $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. As only gonadotrophs exhibiting a single set of morphological characteristics were used in the present study, it is likely that the two native GnRHs stimulate an increase in $[Ca^{2+}]_i$ from the same subpopulation of gonadotrophs. In further support of this view, both sGnRH and cGnRH-II stimulate an increase in $[Ca^{2+}]_i$ in the same cell when applied sequentially at a low dose. The GnRH-induced increases in $[Ca^{2+}]_i$ observed in this subpopulation of gonadotrophs appear to be receptor-mediated. Both GnRHs stimulate a dose dependent increase in $[Ca^{2+}]_i$. In addition, the GnRH-antagonist, analog E, reversibly blocks sGnRH-induced Ca^{2+} responses. Although GnRH receptors have been located in histochemically identified goldfish gonadotrophs (Cook *et al.*, 1991), direct evidence for the presence of GnRH receptors in this subpopulation of morphologically identified goldfish gonadotrophs awaits further study.

Spatiotemporal aspects of GnRH-induced Ca^{2+} response

A variety of temporal patterns in the $[Ca^{2+}]i$ response to GnRH have been observed in different cells expressing GnRH-receptors, including slow-monophasic, transient-spike, oscillatory, biphasic oscillatory and biphasic non-oscillatory (Fig. 6.21A). In goldfish, slow-monophasic, transient-spike and biphasic, non-oscillatory Ca^{2+} responses were observed at varying concentrations of GnRH. Non-oscillatory changes in $[Ca^{2+}]_i$ were also observed in African catfish gonadotrophs (Rebers *et al.*, 1995), αT3-1 (McArdle *et al.*, 1995) and LβT2 (Thomas *et al.*, 1996) clonal gonadotrophs, as well as immortalized GnRH neurons (Krsmanovic, *et al.*, 1993). In these cells, as in goldfish gonadotrophs, GnRH often induced biphasic changes in $[Ca^{2+}]_i$, consisting of an early spike phase followed by a lower amplitude plateau phase. Also, in response to high concentrations of GnRH (≥ 10 nM), normal and ovariectomized rat gonadotrophs exhibited biphasic oscillatory or non-oscillatory changes in $[Ca^{2+}]_i$ (Stojilkovic *et al.*, 1994). Under the experimental conditions used in this study, goldfish gonadotrophs did not exhibit the characteristic, large amplitude $[Ca^{2+}]_i$ oscillations which are observed in rat gonadotrophs in response to lower concentrations (≤ 10 nM) of GnRH. In this regard, the temporal resolution employed in this study may not have been sufficient to resolve Ca^{2+} oscillations in goldfish gonadotrophs.

In different cells expressing GnRH receptors, an increase in GnRH concentration may elicit either an all-or-none increase in $[Ca^{2+}]_i$, a change in the amplitude of $[Ca^{2+}]_i$ or a change in frequency of the cytosolic Ca^{2+} oscillations (Fig. 6.21B; Stojilkovic *et al.*, 1994). The present data demonstrate that increasing concentrations of sGnRH or cGnRH-II induce a corresponding increase in the net change in peak $[Ca^{2+}]_i$ in goldfish gonadotrophs. These data are consistent with an amplitude modulated Ca^{2+} response to GnRH in these cells. A similar response to GnRH has also been observed in αT3-1 (McArdle *et al.*, 1995) and LβT2 (Thomas *et al.*, 1996) clonal gonadotrophs, as well as immortalized GnRH neurons (Krsmanovic, *et al.*, 1993). In contrast, in rat gonadotrophs, irrespective of their developmental stage, physiological condition or sex, increasing concentrations of GnRH does not affect the amplitude of the Ca^{2+} response (Stojilkovic *et al.*, 1993). Instead, the frequency of GnRH-induced Ca^{2+} oscillations are modulated by GnRH concentration. In rat Leydig cells, which also contain GnRH-receptors, all-or-none Ca^{2+} responses were reported in response to increasing concentrations of GnRH (Tomic *et al.*, 1995).

In many different neuronal and endocrine cells, changes in $[Ca^{2+}]_i$ occur in localized regions of the cytoplasm. In the present study, both sGnRH and cGnRH-II evoked a localized increase in $[Ca^{2+}]_i$ in a large proportion of the gonadotrophs tested. Similar responses to GnRH occur in rat gonadotrophs (Rawlings *et al.*, 1991). Several different factors may contribute to the localized Ca^{2+} responses in rat and goldfish gonadotrophs: the diffusion rate of Ca^{2+} through the cytoplasm, the concentration and distribution of mobile Ca^{2+} buffering proteins, as well as the distribution of plasma membrane Ca^{2+} channels, intracellular Ca^{2+} release sites and GnRH receptors. In this respect, activation of voltage-dependent Ca^{2+} channels or inhibition of the endoplasmic reticulum Ca^{2+}-ATPase stimulates a localized increase in $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. This indicates that voltage-dependent Ca^{2+} channels and intracellular Ca^{2+} release sites are clustered in specific regions of the plasma membrane and cytoplasm, respectively. Intracellular Ca^{2+} release sites and voltage-dependent Ca^{2+} channels also appear to be spatially localized in rat gonadotrophs (Rawlings *et al.*, 1991).

Non-polarized increases in $[Ca^{2+}]_i$ were also observed in a small portion of the cells responding to GnRH. These results may indicate that in some cells, there is a more uniform distribution of GnRH-receptors, intracellular Ca^{2+} stores or Ca^{2+} channels in the plasma membrane. The numerous small Ca^{2+}

'hot spots' observed in these cells indicate that there is some degree of clustering of intracellular Ca^{2+} release or entry sites in these cells; however, they may be more dispersed compared to those cells which exhibit polarized Ca^{2+} responses. Alternatively, multiple pools of intracellular Ca^{2+} may be present in these cells, resulting in Ca^{2+}-induced Ca^{2+} release, which may lead to the rapid spread of Ca^{2+} throughout the cytoplasm. To more clearly elucidate the mechanisms underlying the two different spatial patterns of Ca^{2+} signals, it will be necessary to use higher resolution techniques, such as confocal microscopy, in combination with greater temporal resolution.

Mobilization of intracellular Ca^{2+} mediates the acute actions of GnRH

In general, agonist-induced increases in $[Ca^{2+}]_i$ can occur through a change in the release or sequestration rate of Ca^{2+} by cytoplasmic Ca^{2+} stores or by a change in the rate of ionic flux across the plasma membrane. The present data show that acute activation of sGnRH and cGnRH-II receptors mobilize intracellular Ca^{2+} stores to increase $[Ca^{2+}]_i$. At least three lines of evidence support this view. First, inhibition of Ca^{2+} influx by removal of extracellular Ca^{2+} or by addition of inorganic Ca^{2+} channel blockers does not affect GnRH-induced increase in $[Ca^{2+}]_i$. Second, neither sGnRH nor cGnRH-II affect inward currents through voltage-dependent Ca^{2+} channels. Third, thapsigargin-sensitive intracellular Ca^{2+} stores are present in identified goldfish gonadotrophs. Additionally, sGnRH stimulates the production of $InsP_3$ isoforms in dispersed goldfish gonadotrophs (Chang *et al.*, 1995). Production of $InsP_3$ is known to initiate the release of intracellular Ca^{2+} in many cell types, including rat and chicken gonadotrophs (Davidson *et al.*, 1990; Stojilkovic and Catt, 1992; Stojilkovic *et al.*, 1994). Although the present study demonstrates the involvement of intracellular Ca^{2+} stores in the acute actions of sGnRH and cGnRH-II, it does not preclude the involvement of extracellular Ca^{2+} in the maintenance of prolonged GnRH stimulation. For example, prolonged stimulation of GTH-II secretion in goldfish requires Ca^{2+} entry through dihydropyridine-sensitive Ca^{2+} channels (Jobin and Chang, 1992a; Jobin *et al.*, 1996). In rat gonadotrophs, extracellular Ca^{2+} entry is also necessary for sustaining $[Ca^{2+}]_i$ oscillations during prolonged GnRH receptor activation (Stojilkovic *et al.*, 1992).

The apparent lack of extracellular Ca^{2+} involvement in mediating acute GnRH-induced increases in $[Ca^{2+}]_i$ is inconsistent with the proposed involvement of extracellular Ca^{2+} entry in mediating short-term GTH-II release secretion (Jobin *et al.*, 1996). One possible reason for the apparent inconsistencies is that in these hormone release studies, extracellular Ca^{2+} entry was impeded for longer periods of time (60 to 90 min) before GnRH addition, as compared to the 2-min pre-exposure in the present study. This may have depleted intracellular Ca^{2+} stores, which would severely impede GnRH-induced mobilization of stored Ca^{2+}, and hence GTH-II secretion. Results from the present study indicate that Ca^{2+} entry and the activity of the intracellular Ca^{2+} stores are closely related, as discussed earlier. Therefore, it is likely that the dependence on extracellular Ca^{2+} observed during short-term GnRH stimulation of GTH-II secretion reflects the depletion of GnRH releasable pools of intracellular Ca^{2+} following prolonged periods of attenuated Ca^{2+} influx.

Physiological implications and summary

The present results demonstrate that sGnRH and cGnRH-II increase $[Ca^{2+}]_i$ by the mobilization of intracellular Ca^{2+} (Fig. 6.22). Whether GnRH stimulates the mobilization of intracellular Ca^{2+} by the generation of $InsP_3$ or other signaling pathways such as PKC is not known. However, GnRH has been demonstrated to stimulate the production of $InsP_3$, $InsP_2$ and higher InsPs (Chang *et al.*, 1995). Alternatively, PKC has been demonstrated to play a critical role in the regulation of GnRH-stimulated GTH-II secretion and may also participate in the mobilization of intracellular Ca^{2+} (see Chapter 7). Nevertheless, the resulting increase in $[Ca^{2+}]_i$ likely mediates secretion, as similar concentrations of GnRH stimulate GTH-II secretion from dispersed goldfish pituitary cells (Chang *et al.*, 1993; Chang *et al.*, 1996). Although the present study does not implicate the involvement of extracellular Ca^{2+} entry in mediating the acute actions of either sGnRH or cGnRH-II, Ca^{2+} entry may be important in maintaining the intracellular Ca^{2+} stores during prolonged GnRH receptor activation. Extracellular Ca^{2+} entry may also be important for the maintenance of intracellular Ca^{2+} stores in unstimulated gonadotrophs. Modulation of the Ca^{2+} channels by various neuroendocrine factors, such as dopamine (Chapter 5), may regulate the hormone release response by affecting the availability of intracellular Ca^{2+} during stimulation by GnRH.

The functional significance of localized changes in goldfish gonadotrophs is not as clear as that for other cell types. For example, localized changes in $[Ca^{2+}]_i$ rather than uniform increases, have been demonstrated to be important in mediating fast synaptic transmission in neurons (Llinas *et al.*, 1981; Augustine *et al.*, 1987; Zucker, 1993) and are important in restricting the actions of Ca^{2+} to specific regions of the cytoplasm in neuronal growth cones (Silver *et al.*, 1990; Davenport and Kater, 1992). In gonadotrophs, it is possible that local changes in $[Ca^{2+}]_i$ may be necessary to restrict hormone secretion to a particular region of the cell. In this respect, it has been demonstrated that rat gonadotrophs contact blood capillaries at only one point along their cell surface (Keefer and Smith, 1981). Unlike the situation in mammals (Garner and Blake, 1981), aggregation of secretory granules towards any one particular region of the cell have not been reported in goldfish gonadotrophs. It remains to be determined if localized changes in $[Ca^{2+}]_i$ plays a role in limiting the hormone response to a specific pool or region of the cell. As discussed in Chapter 1, cAMP and potential neuroendocrine regulators using the cAMP-dependent signal transduction pathways may utilize GTH-II pools which are distinct from those mobilized during GnRH- and PKC/Ca^{2+}-dependent hormone release (Jobin *et al.*, 1993).

In summary, results reported in this chapter established a single-cell fura-II Ca^{2+}-imaging techinique for studying $[Ca^{2+}]_i$ changes in a teleost gonadotroph. Results also confirmed the involvement of $[Ca^{2+}]_i$ changes in mediating GnRH stimulation of GTH-II release as predicted by earlier pharmacological studies. In addition, the roles of intracellular Ca^{2+} release and extracellular Ca^{2+} influx, as well as temporal and spatial changes in $[Ca^{2+}]_i$ in regulating cell responses will be an important area to pursue in future studies.

Table 6.1. GnRH actions on voltage-dependent Ba^{2+} currents elicited by a 40 msec voltage-step to +10 mV from a holding potential of -80 mV.

	Peak Ba^{2+} current amplitude (pA)	
Treatment	Control	GnRH
100 nM sGnRH (*n* = 3)	-114.1 ± 31.5	-116.1 ± 36.0[a]
100 nM cGnRH-II (*n* = 7)	-266.1 ± 83.6	-245.2 ± 88.1[a]

[a] Denotes similar values compared to controls (paired *t* -test, $P > 0.05$). All experiments were performed with gonadotrophs prepared form sexually mature goldfish.

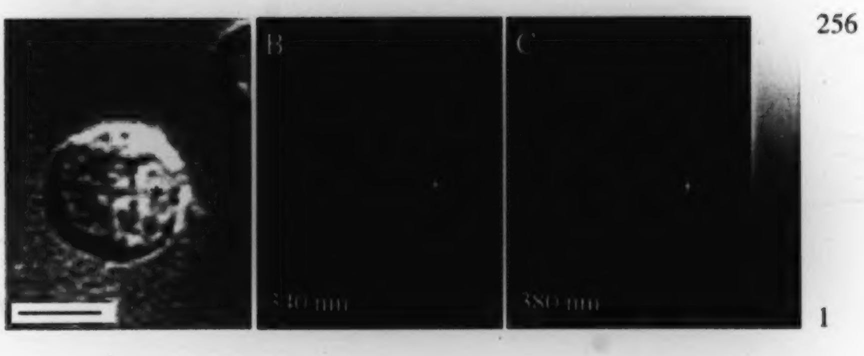


Fig. 6.1. Typical example of an identified goldfish gonadotroph loaded with Fura-II/AM. A. DIC photomicrograph of an identified goldfish gonadotroph. B and C. Epifluorescent images of fluorescent intensities emitted at 510 nm following alternate excitation at 340 nm (B) and 380 nm (C) from the cell shown in (A). It was loaded with 10 µM fura-II/AM and 25 µg/ml pluronic F-127 for 1 hr at 27 °C. The position of the nucleus is indicated (*asterisks*). Gray-scale bar represents the fluorescent intensity scale from 1 - 256. Bar: 10 µm.

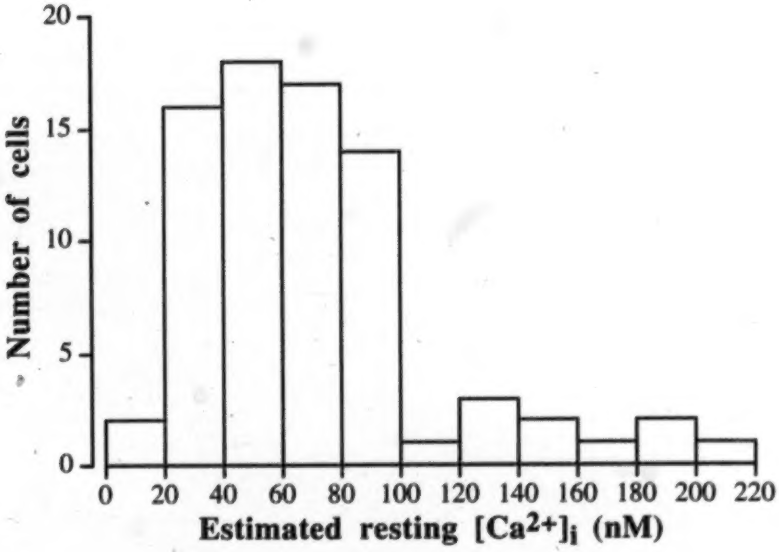


Fig. 6.2. Distribution of resting $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. Gonadotrophs were bathed in fura-II medium containing 5 mM Ca^{2+} ($n = 76$).

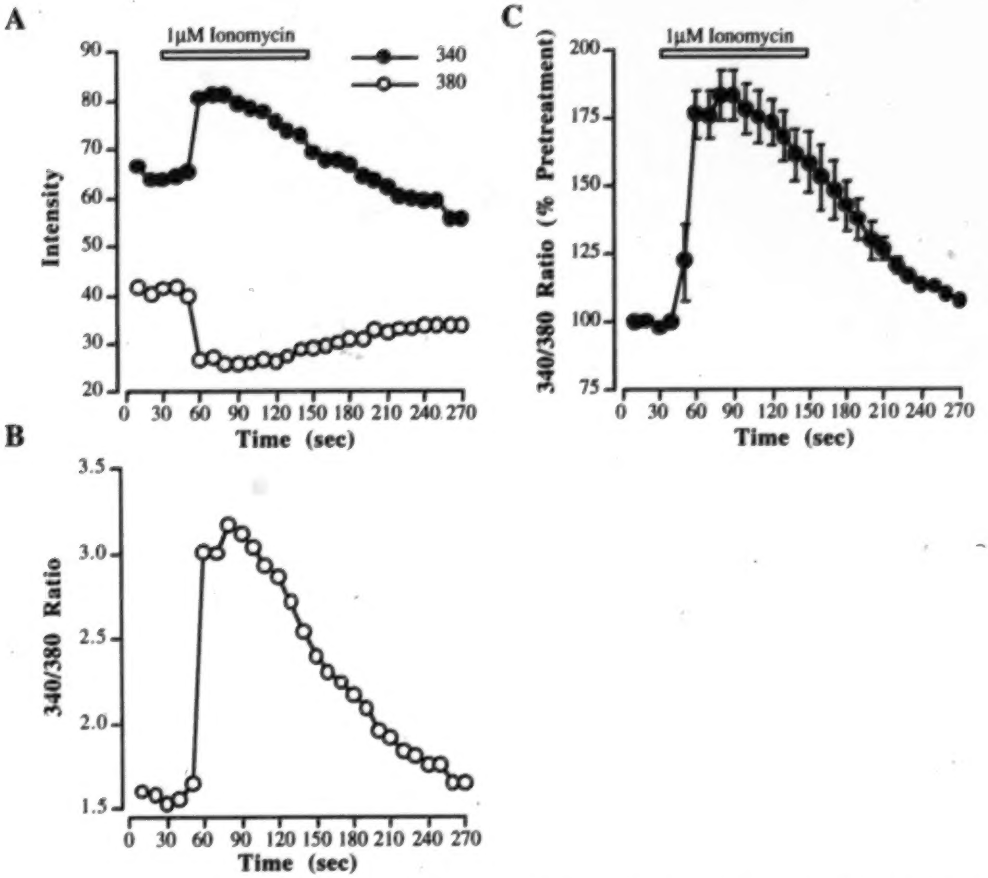


Fig. 6.3. Effects of ionomycin on $[\mathrm{Ca}^{2+}]_i$ in identified goldfish gonadotrophs. Gonadotrophs were exposed to a 2-min application of 1 μM ionomycin (*bar*). A. Dual wavelength measurements of fluorescence intensity measured 510 nm following excitation at 340 and 380 nm. Measurements were taken in 10-sec intervals. Intensity of fluorescence was measured on a digital scale of 256 levels. B. The fluorescence response expressed as 340/380 ratio of the same cell as in (A). C. Cumulative results were normalized as a percentage of pretreatment 340/380 ratio values (1.48 ± 0.07) and presented as mean ± SEM ($n = 3$).

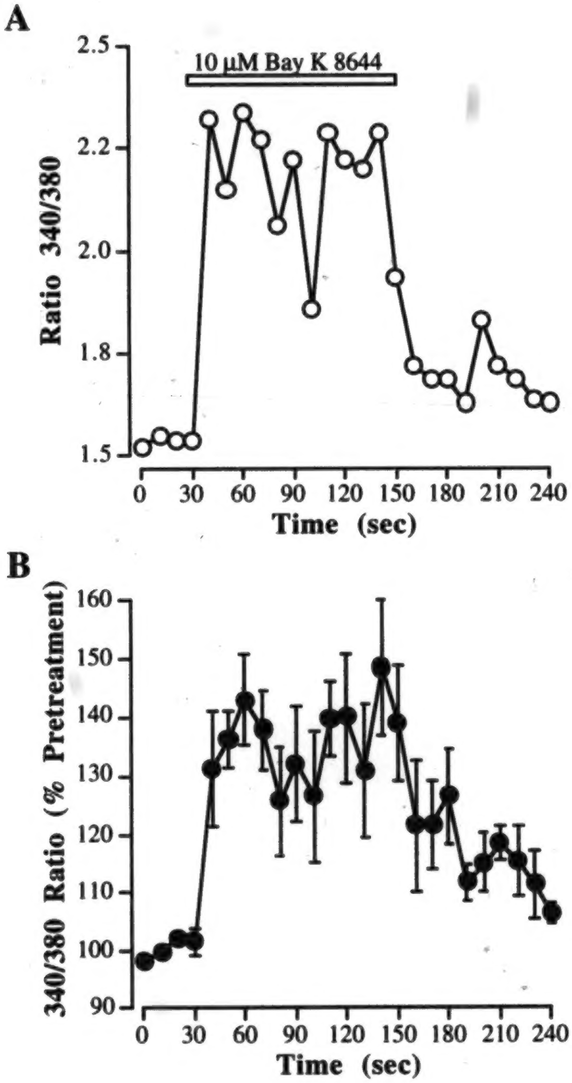


Fig. 6.4. Effects of S(-)-Bay K 8644 on $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. Gonadotrophs were exposed to a 2-min application of 10 μM S(-)-Bay K 8644 (*bar*). A. The fluorescence response expressed as a 340/380 ratio from a single gonadotroph. Measurements were taken in 10-sec intervals. B. Cumulative results from separate experiments were normalized as a percentage of pretreatment 340/380 ratio values (1.45 ± 0.06) and presented as mean ± SEM ($n = 4$).

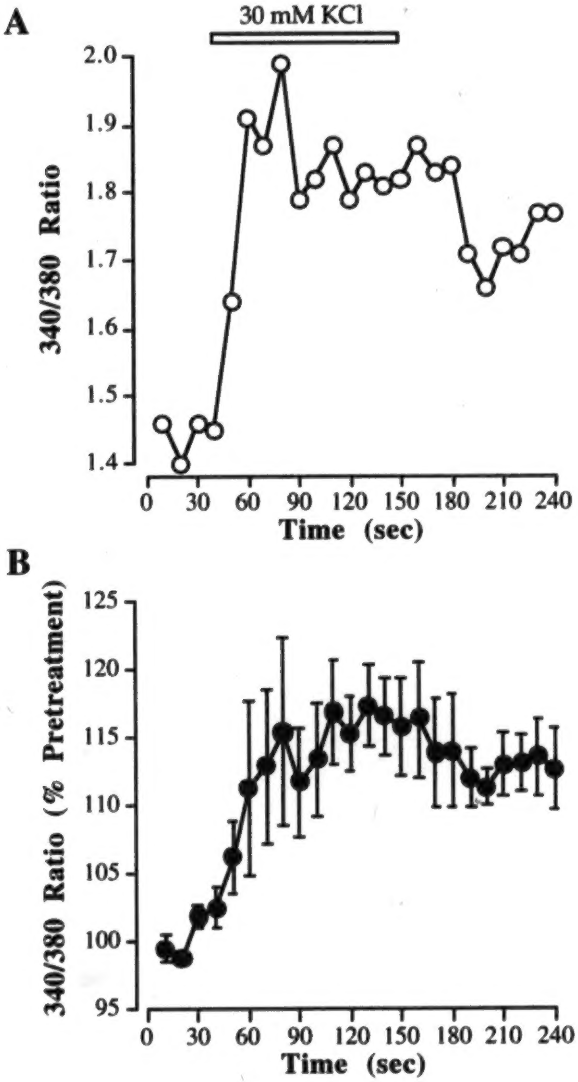


Fig. 6.5. Effects of a high concentration of extracellular K^+ on $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. Gonadotrophs were exposed to a 2-min application of 30 mM extracellular KCl (*bar*). A. The fluorescence response expressed as a 340/380 ratio from a single gonadotroph. Measurements were taken in 10-sec intervals. B. Cumulative results from separate experiments were normalized as a percentage of pretreatment 340nm/380nm ratio values (1.21 ± 0.04) and presented as mean ± SEM (*n* = 4).

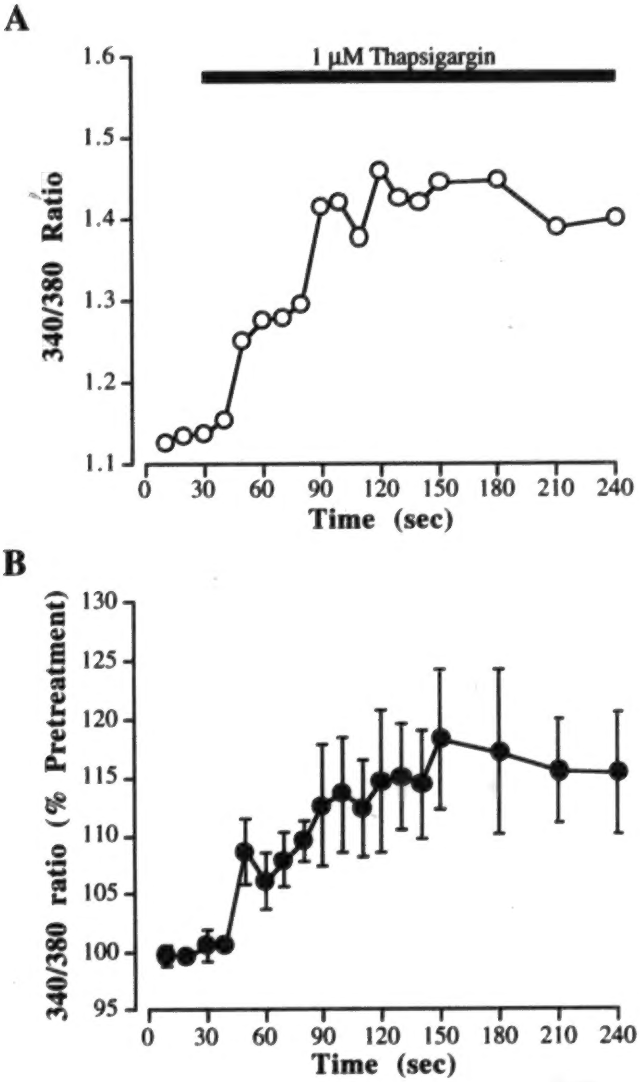


Fig. 6.6. Effects of thapsigargin on $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. Gonadotrophs were exposed to a 2-min application of 1 µM thapsigargin (*bar*). A. The fluorescence response expressed as a 340/380 ratio from a single gonadotroph. Measurements were taken in 10-sec intervals. B. Cumulative results from separate experiments using gonadotrophs prepared from sexually regressed goldfish were normalized as a percentage of pretreatment 340nm/380 nm ratio values (1.13 ± 0.04) and presented as mean ± SEM (*n* = 4).

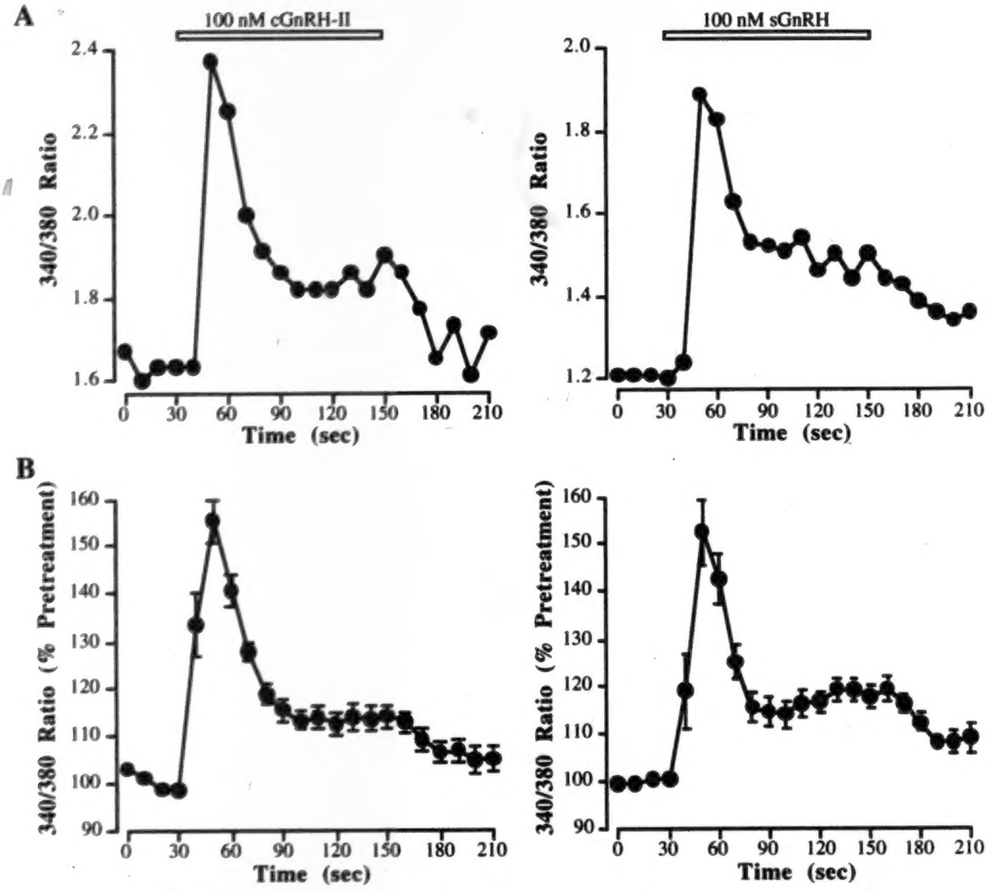


Fig. 6.7. Effects of sGnRH and cGnRH-II on $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. Gonadotrophs were exposed to either 100 nM cGnRH-II (*left panel:* n = 15) or 100 nM sGnRH (*right panel:* n = 8) for 2 min (*bar*). A. The fluorescence response expressed as a 340/380 ratio from a single gonadotroph. Measurements were taken in 10-sec intervals. B. Cumulative results from separate experiments were normalized as a percentage of pretreatment 340nm/380nm ratio values prior to the application of cGnRH-II (1.33 ± 0.05) or sGnRH (1.28 ± 0.04) and presented as mean ± SEM.

Fig. 6.8. Specificity of GnRH action on $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. Pseudo-color image of the 340/380 ratio of a gonadotroph (*G*) and a unidentified cell (*U*) loaded with fura-II/AM before (A) and during (B) the application of 100 nM cGnRH-II. Bar: 10 μm.

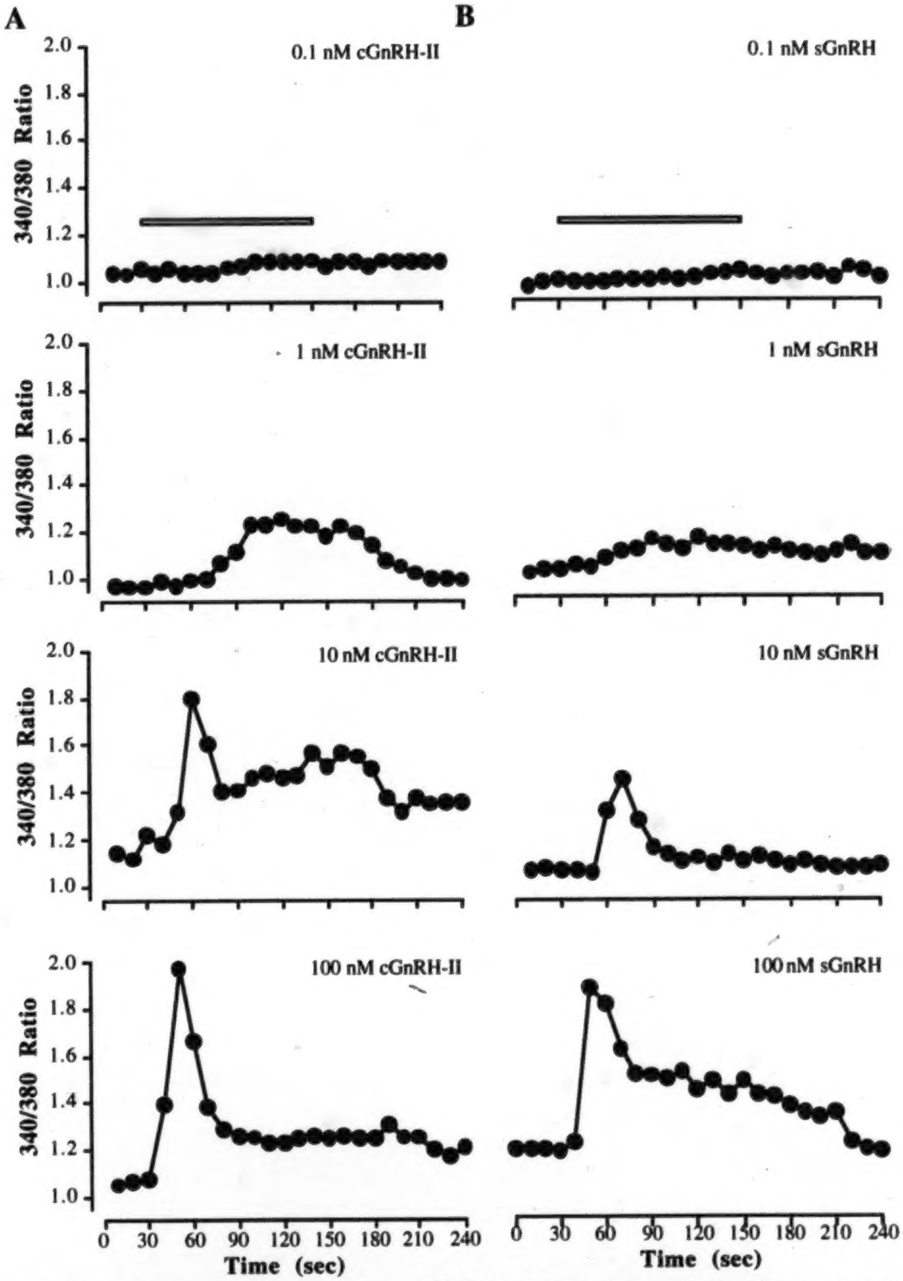


Fig. 6.9. Temporal patterns in $[Ca^{2+}]_i$ elicited by application of different concentrations of sGnRH and cGnRH-II. Typical examples of the temporal patterns in the cytosolic Ca^{2+} responses to a 2-min application (*Bar*) of 0.1, 1, 10 and 100 nM cGnRH-II (A) or sGnRH (B). Data from separate gonadotrophs are presented as the 340/380 ratio collected every 10 sec.

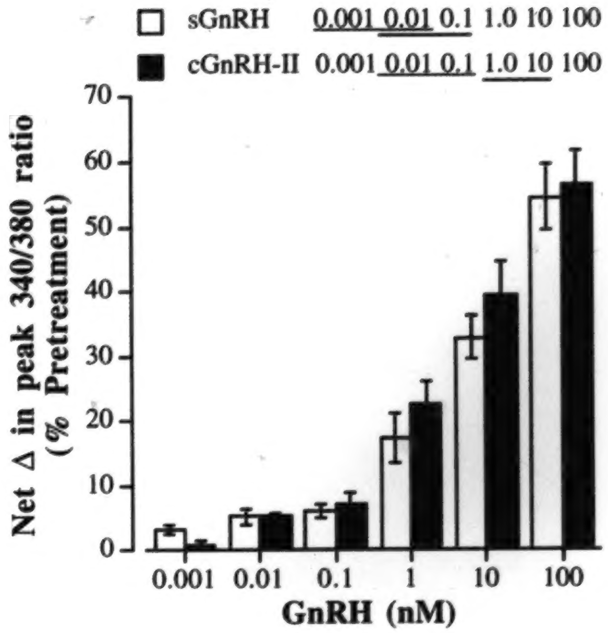


Fig. 6.10. cGnRH-II and sGnRH induced dose-dependent increases in $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. Cumulative results from separate experiments were normalized by calculating the maximal rise in $[Ca^{2+}]_i$ as a percentage of pretreatment 340/380 ratio values prior to the application of cGnRH-II (1.12 ± 0.06) or sGnRH (1.14 ± 0.05), and are presented as mean ± SEM (n = 3 - 15). Treatment groups having similar responses ($P > 0.05$; Kruskal-Wallis test followed by Mann-Whitney U test) are identified by the same underscore.

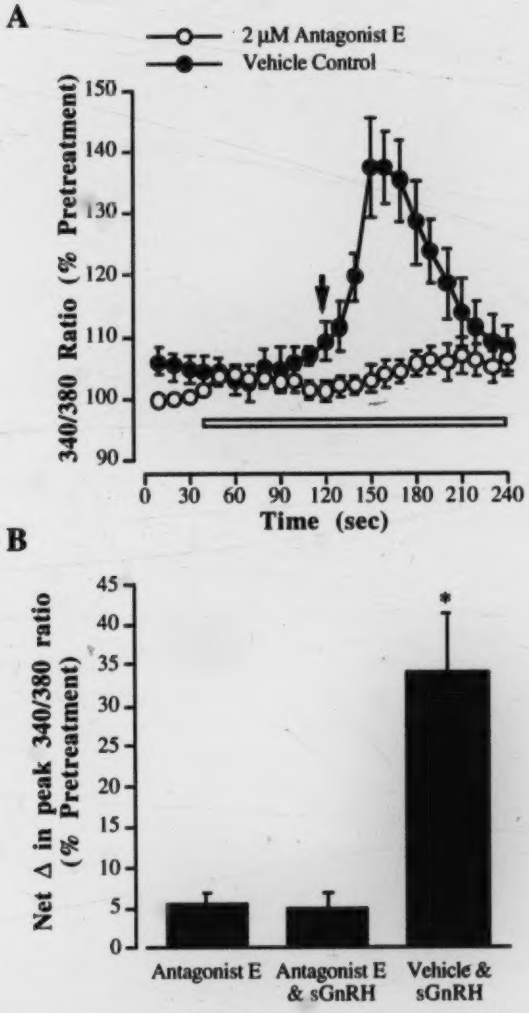


Fig. 6.11. Effects of GnRH antagonist E on sGnRH-induced increases in $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. A. Superimposed traces from gonadotrophs exposed to sequential pulses of 100 nM sGnRH (*arrow*) in the presence and absence of 2 μM antagonist E (*Bar*). A 5-min wash period was given between sequential treatments on the same cells. Results from separate experiments were normalized as a percentage of pretreatment 340/380 ratio values prior to the application of antagonist E (1.10 ± 0.04). Values in (A) and (B) are presented as mean ± SEM ($n = 6$). *Asterisk* denote significant differences between each group ($P < 0.05$; ANOVA followed by Fisher's LSD test). All experiments were performed with gonadotrophs prepared from sexually regressed goldfish.

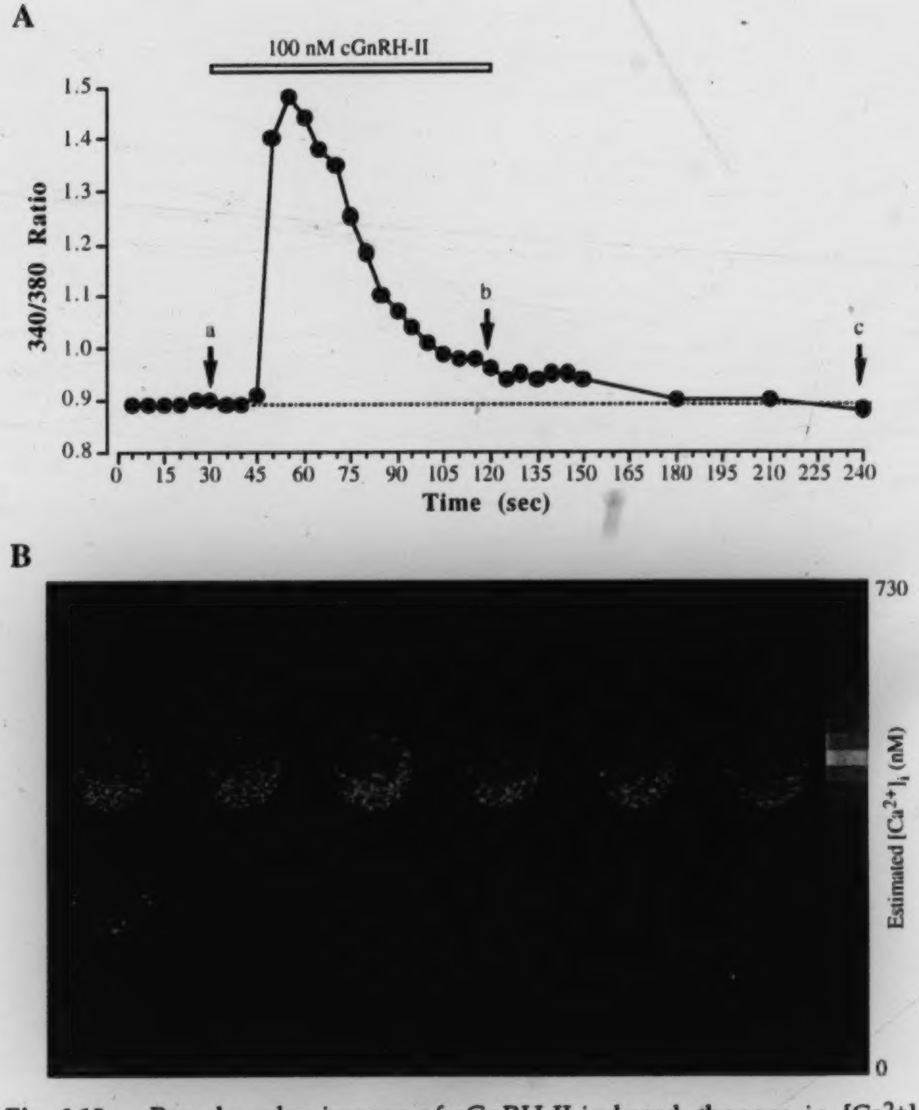


Fig. 6.12. Pseudo-color images of cGnRH-II-induced changes in $[Ca^{2+}]_i$ in a single identified gonadotroph. A. Temporal pattern of 100 nM cGnRH-II-induced (*bar*) changes in 340/380 ratio. Ratio values were collected every 5 sec. B. A sequential series of pseudo-color ratio images illustrating a polarized increase in $[Ca^{2+}]_i$ in response to 100 nM cGnRH-II. Corresponding ratio values and images in A and B, respectively, are indicated by a - c. Bar: 10 µm.

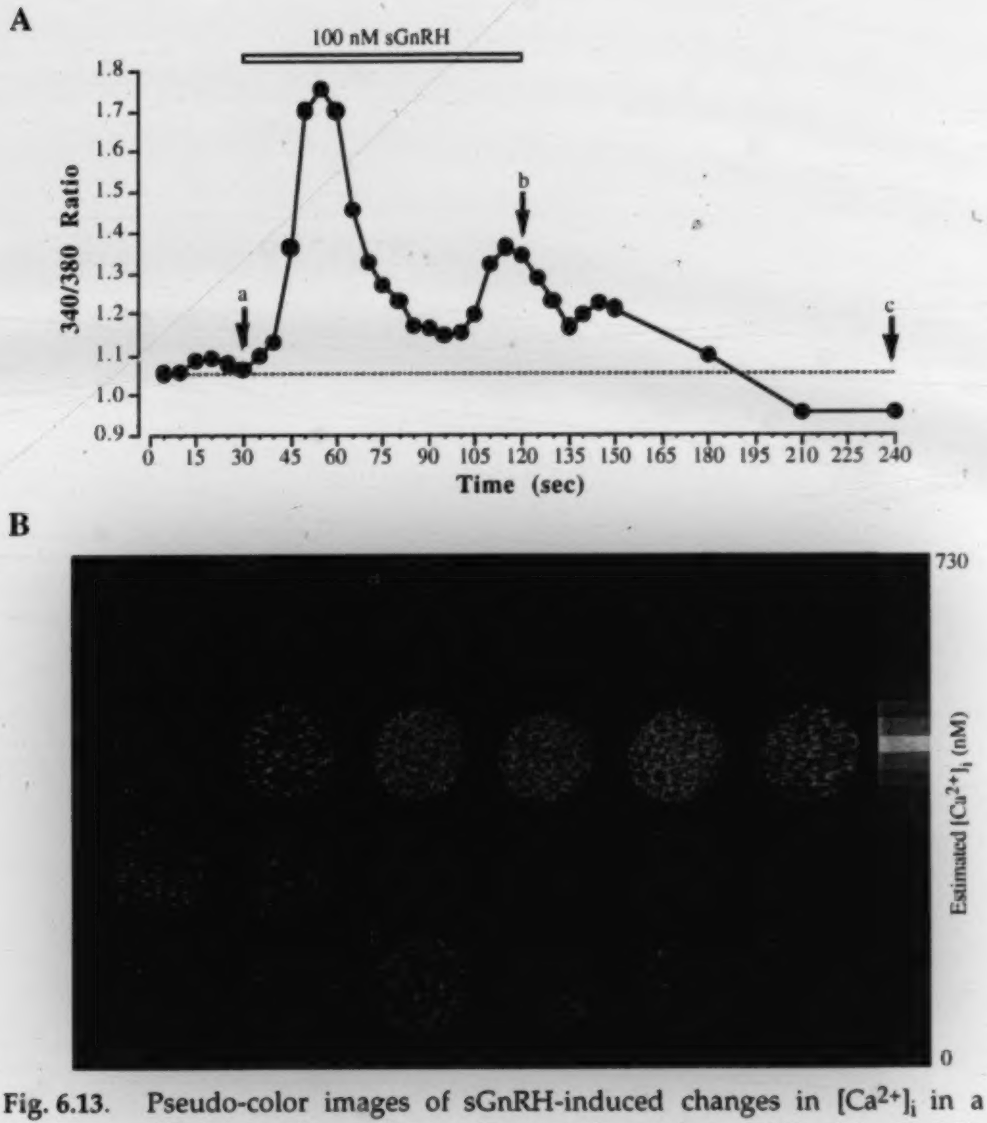


Fig. 6.13. Pseudo-color images of sGnRH-induced changes in $[Ca^{2+}]_i$ in a single identified gonadotroph. A. Temporal pattern of 100 nM sGnRH-induced (*bar*) changes in 340/380 ratio. Ratio values were collected every 5 sec. B. Sequential series of pseudo-color ratio images illustrating a non-polarized increase in $[Ca^{2+}]_i$ in response to sGnRH. Corresponding ratio values and images in A and B, respectively, are indicated by a - c. Bar: 10 µm.

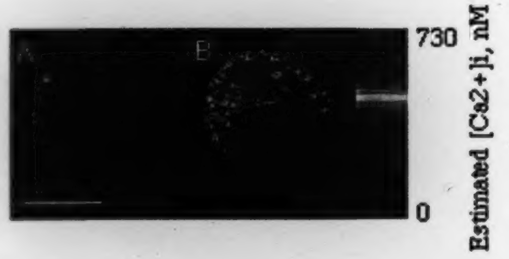


Fig. 6.14. Pseudo-color images of thapsigargin-induced increases in $[Ca^{2+}]_i$ in a single identified gonadotroph. Ratio images of a gonadotroph before (A) and during (B) the application of 1μM thapsigargin. All experiments were performed with gonadotrophs prepared from prespawning goldfish. Bar: 10 μm.

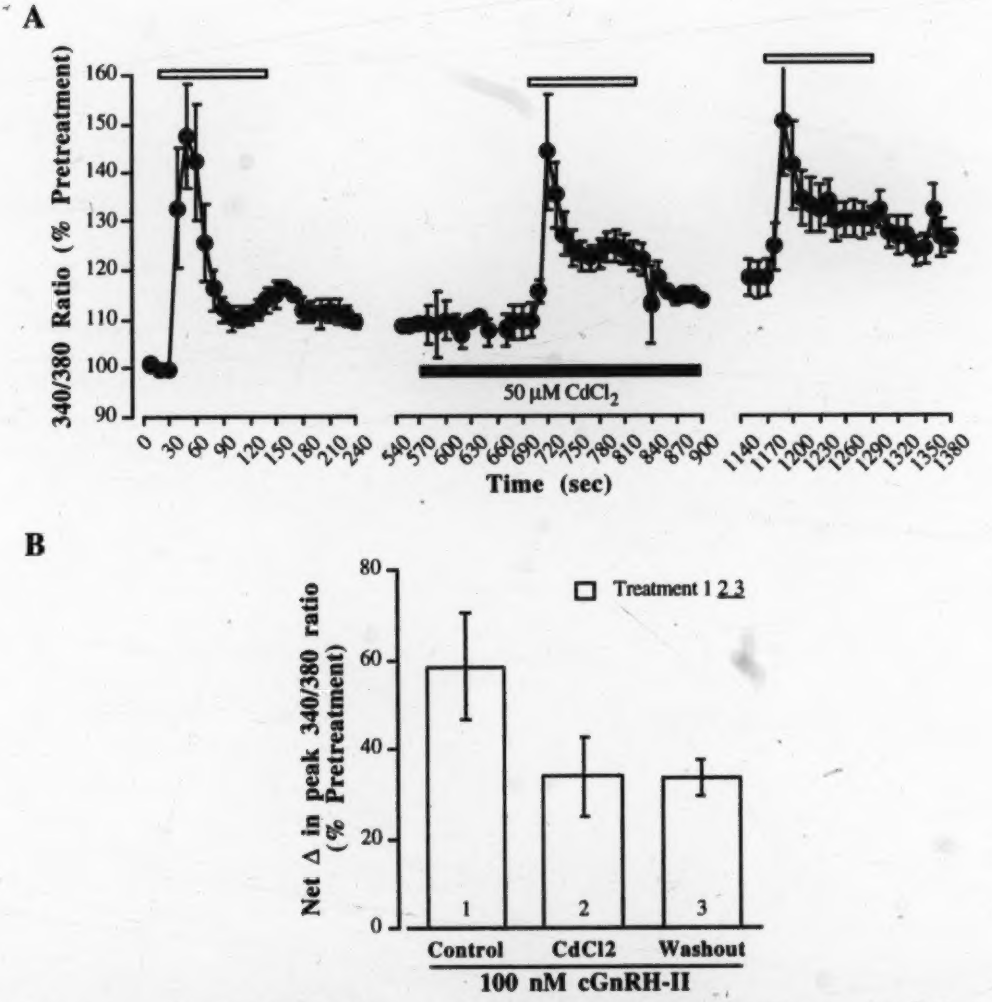


Fig. 6.15. Effects of $CdCl_2$ on cGnRH-II-induced increases in $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. A. $[Ca^{2+}]_i$ response to a 2-min application of 100 nM cGnRH-II (*open bar*) in normal and 50 µM $CdCl_2$-containing medium (*solid bar*). Results from separate experiments were normalized as a percentage of pretreatment 340/380 ratio values prior to the application of the first cGnRH-II pulse (1.03 ± 0.01). B. Net change in peak $[Ca^{2+}]_i$ in response to 100 nM cGnRH-II before, during and after the application of 50 µM $CdCl_2$. Results were normalized as a percentage of pretreatment 340 nm/380 nm ratio values prior to the application of each pulse of cGnRH-II. Treatment groups having similar Ca^{2+} responses ($P > 0.05$, by ANOVA-repeated measures followed by paired *t*-test) are identified by the same underscore. Values in (A) and (B) are presented as mean ± SEM ($n = 6$). All experiments were performed with gonadotrophs prepared from prespawning goldfish.

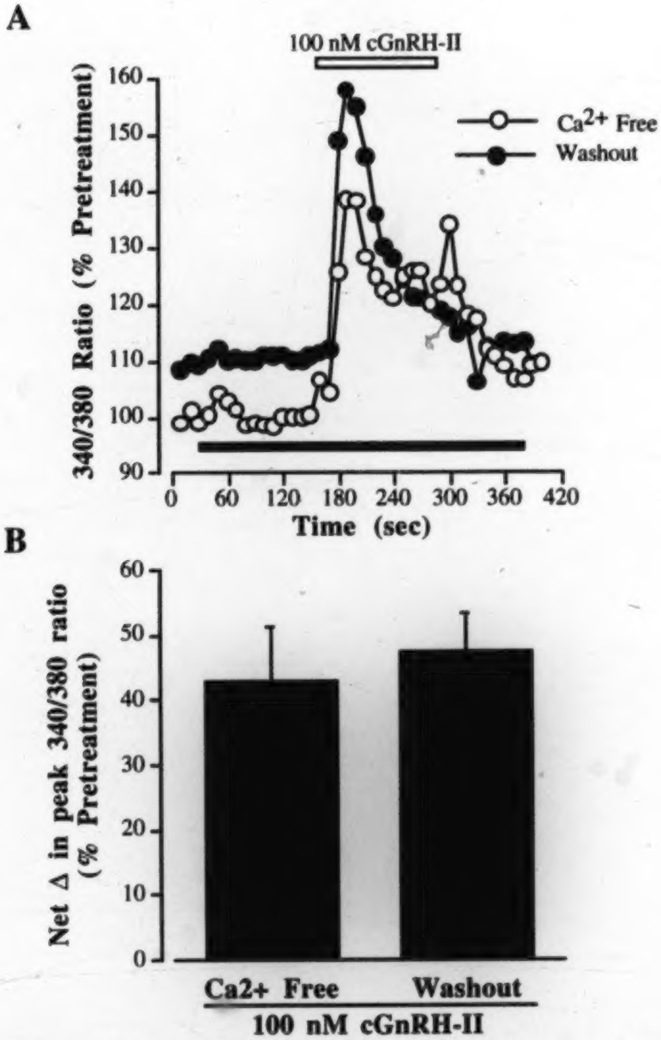


Fig. 6.16. Effects of removal of extracellular Ca^{2+} on cGnRH-II-induced increases in $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. A. Superimposed traces from gonadotrophs exposed to sequential applications of 100 nM cGnRH-II (*bar*) in the presence of Ca^{2+} free medium (*filled bar*) and following the re-addition of 5 mM Ca^{2+} containing medium (Washout). A 7-min wash period was given following the re-addition of Ca^{2+} to the medium before the second application of cGnRH-II. Results from separate experiments were normalized as a percentage of pretreatment 340/380 ratio values prior to the removal of extracellular Ca^{2+} (1.35 ± 0.04). B. Net change in peak $[Ca^{2+}]_i$ as a percentage of 340/380 ratio values prior to the addition of 100 nM cGnRH-II. Values in (A) and (B) are presented as mean ± SEM ($n = 3$).

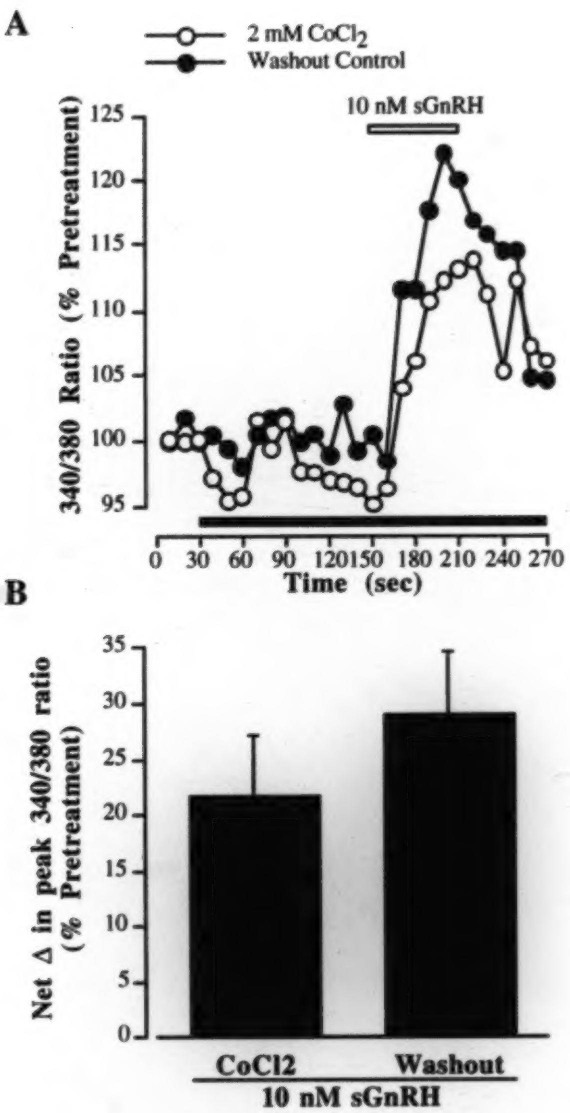


Fig. 6.17. Effects of $CoCl_2$ on sGnRH-induced increases in $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. A. Superimposed traces from gonadotrophs exposed to sequential applications of 10 nM sGnRH (*bar*) in the presence of 2 mM $CoCl_2$ (*filled bar*) and 7 min following the removal of $CoCl_2$ (*washout*). Results from separate experiments were normalized as a percentage of pretreatment 340/380 ratio values prior to the addition of $CoCl_2$ (1.18 ± 0.02; mean ± SEM; $n = 5$). B. Net change in peak $[Ca^{2+}]_i$ as a percentage of 340/380 ratio values prior to the addition of 10 nM sGnRH. Gonadotrophs were prepared from sexually regressed goldfish.

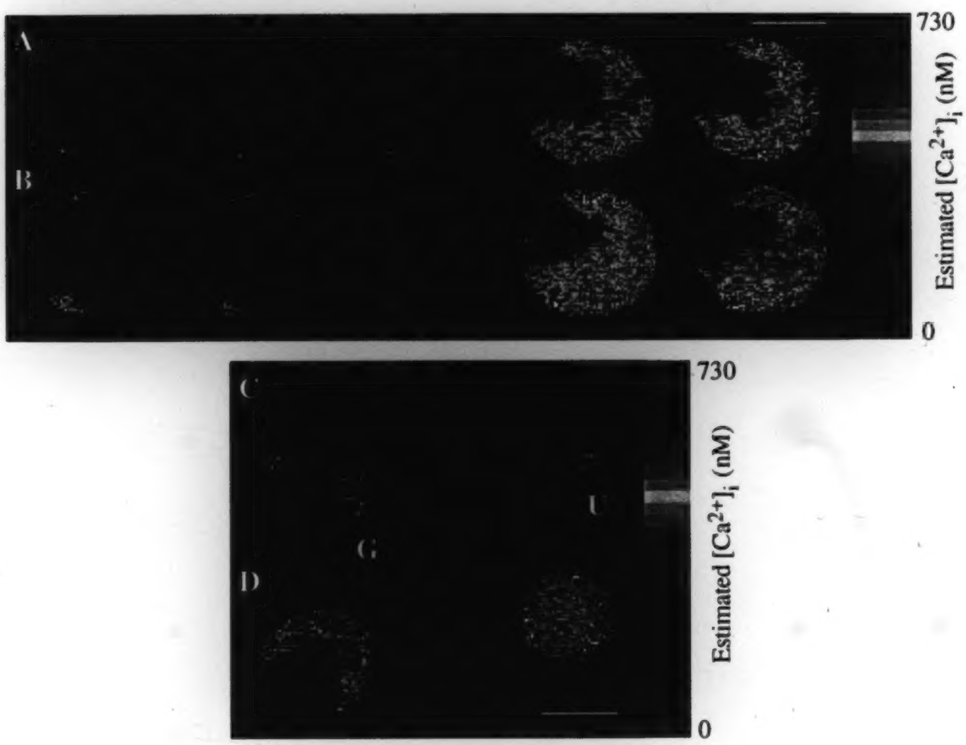


Fig. 6.18. Pseudo-color images of Bay K 8644- and KCl-induced increases in $[Ca^{2+}]_i$ in a single identified gonadotroph. A series of pseudo-color ratio images taken every 10 sec illustrating the local changes in $[Ca^{2+}]_i$ in response to sequential application of 100 nM cGnRH-II (A) and 10 µM Bay K 8644 (B) in the same cell. B. Pseudo-color ratio images of a gonadotroph (G) and an unidentified cell (U) before (C) and during (D) the application of 30 mM KCl. All experiments were performed with gonadotrophs prepared from prespawning goldfish. Bar: 10 µm.

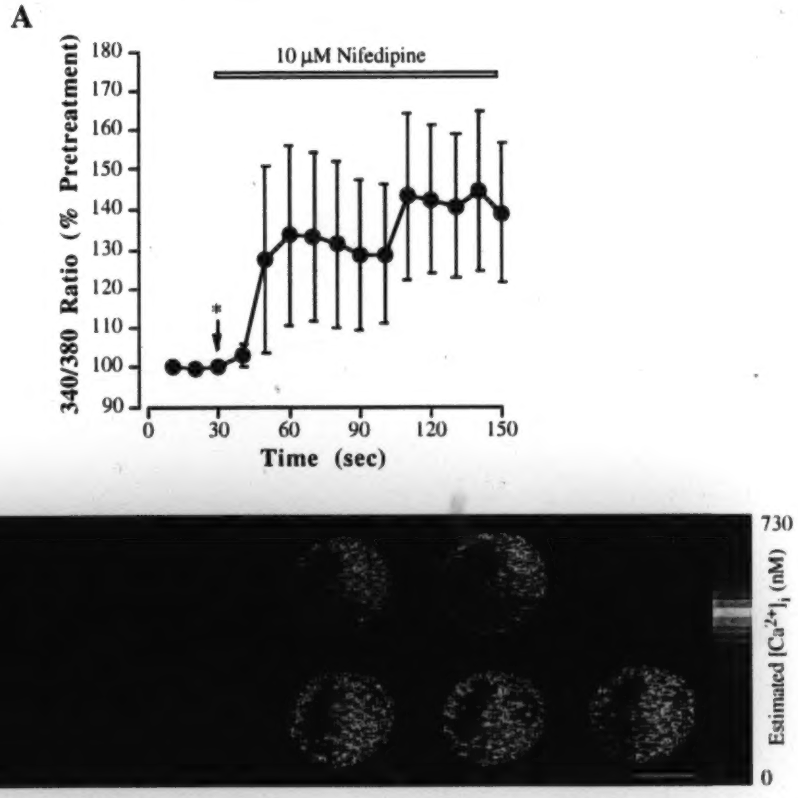


Fig. 6.19. Nifedipine-induced increase in $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. Gonadotrophs were exposed to 10 μM nifedipine for 2 min (*bar*). A. Cumulative results were normalized as a percentage of pretreatment 340/380 ratio values (1.18 ± 0.06) and presented as mean ± SEM ($n = 4$). B and C. A series of pseudo-color ratio images taken every 10 sec illustrating the local changes in $[Ca^{2+}]_i$ in response to sequential application of 10 nM cGnRH-II (B) and 10 μM nifedipine (C) in the same cell. *Asterisk* indicate the time of GnRH or nifedipine application. Bar: 10 μm. All experiments were performed with gonadotrophs prepared from prespawning goldfish.

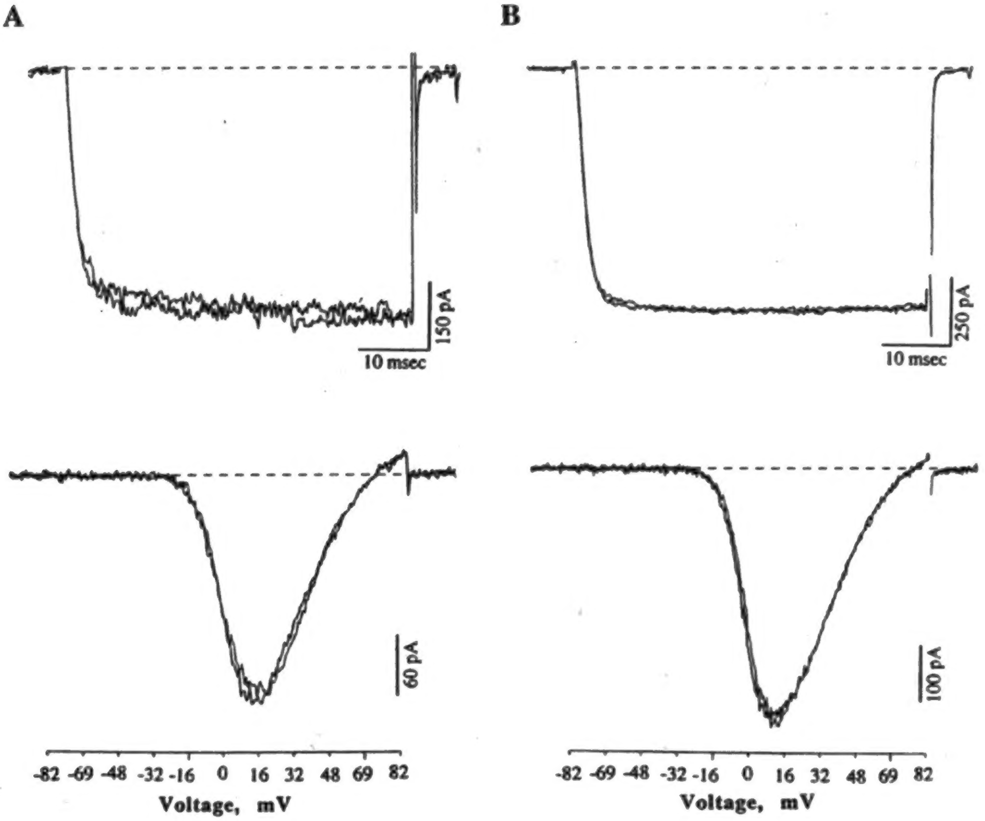


Fig. 6.20. Effects of sGnRH and cGnRH-II on voltage-dependent Ba^{2+} currents in identified goldfish gonadotrophs. A. Upper panel: superimposed Ba^{2+} current traces in the presence or absence of 100 nM sGnRH. Currents were elicited during a 40-msec voltage step to +10 mV (holding potential = -80 mV). Lower panel: current-voltage relationship of the Ba^{2+} current evoked during a 200-msec voltage-ramp from -80 mV to +80 mV in the presence or absence of 100 nM sGnRH-II. B. Upper panel: superimposed Ba^{2+} current traces in the presence or absence of 100 nM cGnRH-II. Currents were elicited during a 40-msec voltage step to +10 mV (holding potential = -80 mV). Lower panel: current-voltage relationship of the Ba^{2+} current evoked during a 200-msec voltage-ramp from -80 mV to +80 mV in the presence or absence of 100 nM cGnRH-II.

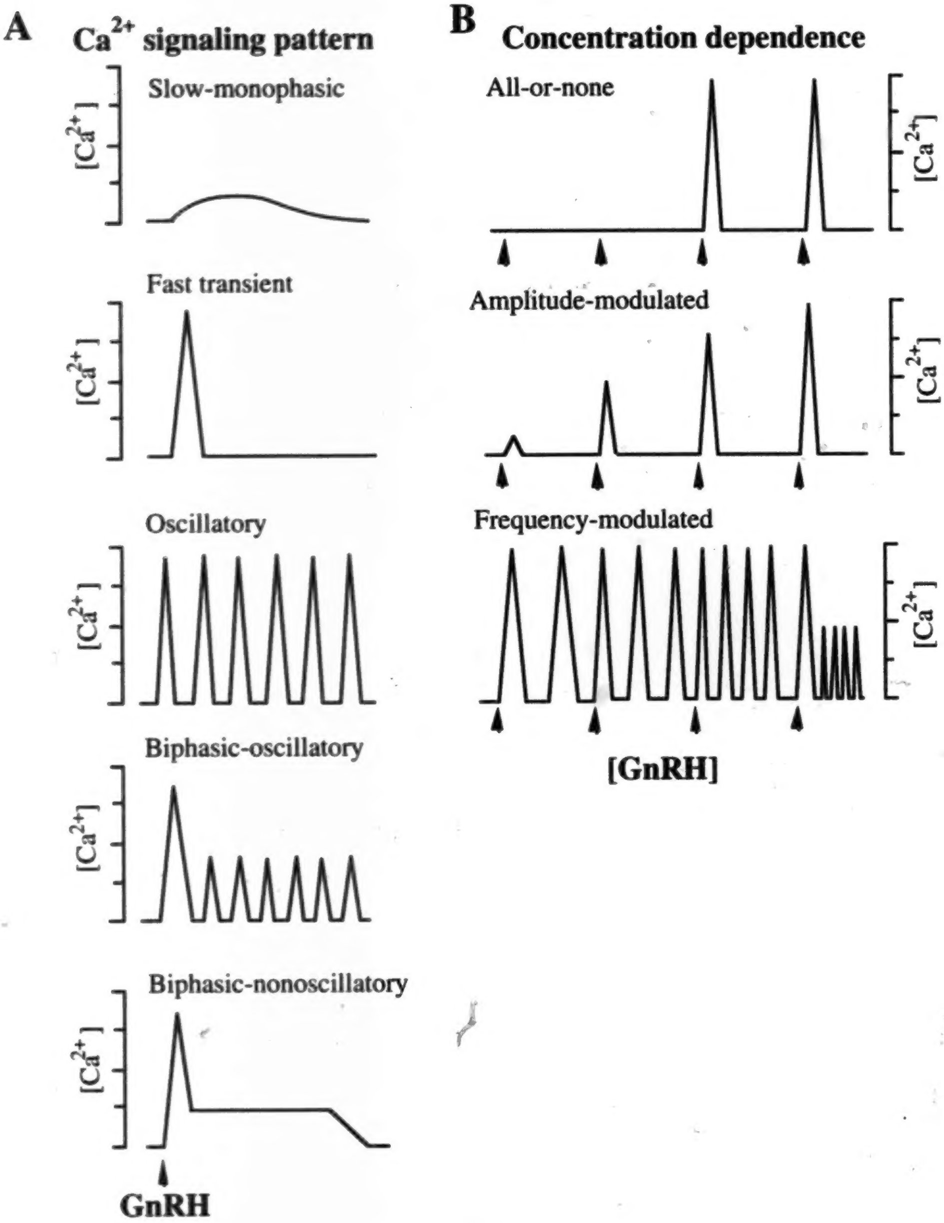


Fig. 6.21. Comparison of the Ca^{2+} signaling patterns (A) and concentration dependence (B) observed in different cell types expressing GnRH receptors. Adapted from Stojilkovic and Catt, 1992; Stojilkovic *et al.*, 1994 and Tomic *et al.*, 1995.

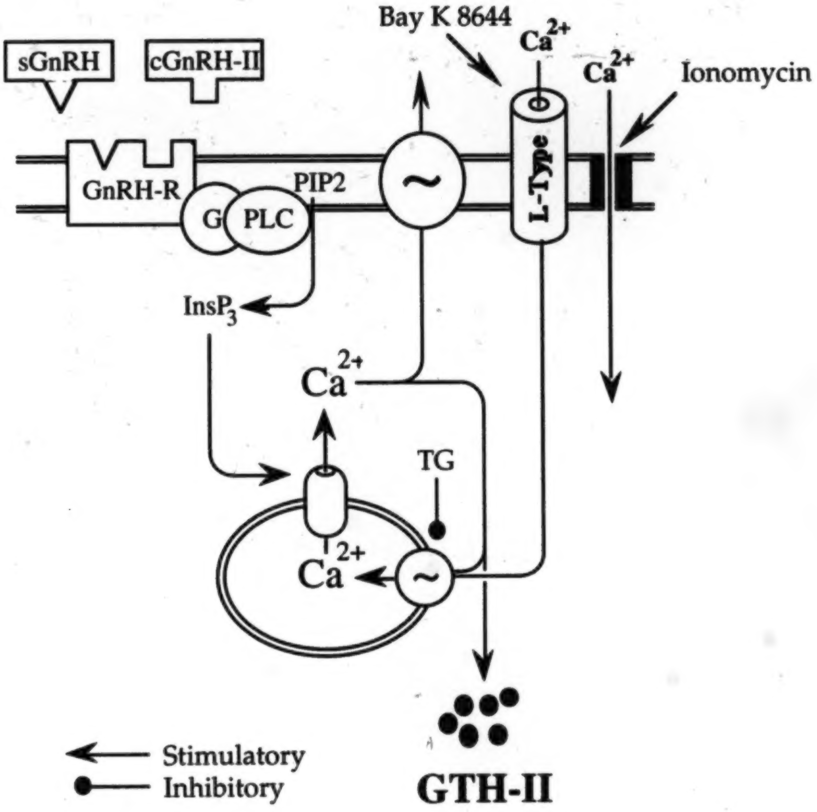


Fig. 6.22. Summary of GnRH-induced increases in $[Ca^{2+}]_i$. Abbreviations: TG = thapsigargin, R = receptor, G = G-protein, PLC = Phospholipase C, PIP = phosphatidylinositol-bisphosphate ($PI[4, 5]P_2$), Inositol (1, 4, 5)-trisphosphate, ~ = Ca^{2+} pump.

Chapter 7

INTERACTIONS BETWEEN PROTEIN KINASE C AND CA^{2+} IN GOLDFISH GONADOTROPHS.

INTRODUCTION

The involvement of PKC in mediating the actions of GnRH in mammalian gonadotrophs has been the subject of much debate (Stojilkovic *et al.*, 1994). Some have suggested that PKC is not involved in the stimulation of LH secretion (McArdle *et al.*, 1987), while others have shown that PKC modulates GnRH action (Davidson *et al.*, 1990; Stojilkovic *et al.*, 1991; Stojilkovic and Catt, 1992; Tse *et al.*, 1995). In contrast, it is clear that PKC plays a prominent role in sGnRH and cGnRH-II stimulated GTH-II secretion from goldfish gonadotrophs (reviewed in Chapter 1). In goldfish, hormone release studies have suggested that PKC-stimulated GTH-II secretion is dependent on Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Jobin and Chang, 1992b; Chang *et al.*, 1996). Activation of PKC stimulates an increase in [Ca^{2+}]$_i$ in mixed populations of dispersed goldfish pituitary cells in both an extracellular and intracellular Ca^{2+} dependent manner (Jobin and Chang., 1992b). These observations indirectly indicate that PKC-dependent GTH-II secretion is in part due to the activation of voltage-dependent Ca^{2+} channels and the subsequent increase in [Ca^{2+}]$_i$.

To more directly test the hypothesis that PKC alters [Ca^{2+}]$_i$, single-cell Ca^{2+} imaging experiments were conducted on morphologically identified goldfish gonadotrophs. In addition, perforated-patch-clamp recording techniques were used to investigate the effects of PKC activation on voltage-dependent Ca^{2+} channels. In an attempt to relate the results from single-cell Ca^{2+} imaging and electrophysiological experiments to secretion, the effects of PKC on voltage-dependent Ca^{2+} channel-mediated GTH-II secretion were monitored in rapid-fraction perifusion studies. The tumor-promoting phorbol ester, tetradecanoyl phorbol-13-acetate (TPA), and the synthetic diacylglycerol, dioctanoyl glycerol (DiC8), were used to activate PKC. The inactive analog of TPA, 4α-phorbol 12, 13 didecanoate (4α-PDD), was used as a negative control for the actions of TPA. In general, the preliminary findings of this study indicate that acute (2 min) activation of PKC stimulates a localized increase in $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. This response does not appear to result directly from an increase in Ca^{2+} entry through voltage-dependent Ca^{2+} channels; however, prolonged activation of PKC may enhance voltage-dependent Ca^{2+} channel activity and/or Ca^{2+} dependent exocytosis.

MATERIALS AND METHODS

General

Common goldfish (*Carassius auratus*; 8-13 cm in body length) were purchased from Grassyforks Fisheries (Martinsville, IN, USA) or Ozark Fisheries (Stoutland, MO, USA) and maintained as previously described (Chapter 5). Pituitaries from sexually regressed and prespawning male and female goldfish were excised and their pituitary cells were dispersed and cultured as previously described (Chang *et al.*, 1990a; Chapter 5). For single-cell Ca^{2+} imaging and patch-clamp experiments, goldfish gonadotrophs were identified on the basis of their unique morphological characteristics as previously described (Chapter 2).

Cell column perifusion studies of GTH-II responses

For the measurement of acute hormone release responses to TPA and DiC8, cell column perifusion studies were performed as previously described (Chang *et al.*, 1990b; Chapter 4). Prior to each experiment, cells were perifused for 4 hr with testing medium at a rate of 15 ml/hr, after which a relatively stable basal secretion rate was established. Experiments began with the collection of 5-min fractions of perifusate for 40 min. This was followed by the collection of 30-sec fractions of perifusate for 20 min, after which the perifusate was collected as 5-min fractions for the remainder of the experiment. Treatment with TPA, DiC8, 4α-PDD or DMSO (vehicle control) began 20 min after the beginning of an experiment and continued for the remainder of the experiment. A 5 min pulse of 30 mM K^+ was given 40 min into the experiment. There was a time delay of 6 to 7 min between the beginning of the drug application and the appearance of the GTH-II response. This delay can be entirely accounted for by the time required for the perifusate to pass through the dead-space (≈1.7 ml) inherent in the perifusion system. Perifusates were kept frozen at -20 °C until their GTH-II contents could be measured using a RIA-specific GTH-II (Peter *et al.*, 1984; Van Der Kraak *et al.*, 1992).

GTH-II content in individual columns was normalized as a percentage of the average pretreatment values obtained in the first four fractions (% Pretreatment). The hormone responses were quantified by determining the net change in GTH-II levels (*i.e.*, area under the curve) and expressed as a percentage of pretreatment values as previously described (Wong *et al.*, 1992; Chapter 4). All values in the text and figures are reported as mean ± SEM. Differences between groups were considered to be significant when $P < 0.05$ using unpaired *t* -test or ANOVA, followed by LSD difference test.

For experiments in which extracellular ion concentrations were modified, a M-199 with Hank's salts prepared without the addition of NaCl or $CaCl_2$ was used as a testing medium (courtesy of Dr. S.S. Stojilkovic, ERRB, NICHD, National Institutes of Health, Bethesda, MD). Na^+ and Ca^{2+} levels were adjusted as required (see below). Under control conditions, the external medium contained (in mM), 136 NaCl, 5.4 KCl and 1.26 $CaCl_2$. For experiments using high concentrations of extracellular K^+, 25 mM NaCl was replaced with equimolar KCl, giving a final extracellular K^+ concentration of 30 mM. All solutions were supplemented with 2.2 g/l $NaHCO_3$, 25 mM HEPES, 100 000 U/l penicillin, 100 mg/l streptomycin, 0.1% BSA; pH was adjusted to 7.2 with TrisCl.

Measurement of $[Ca^{2+}]_i$.

Alterations in $[Ca^{2+}]_i$ were determined using the Ca^{2+} indicator, fura-II (Tsien *et al.*, 1985), as previously described for goldfish gonadotrophs (Chapter 6). Briefly, dispersed pituitary cells were incubated for 60 min at 28 °C with 10 μM fura-II/AM and 25 μg/ml pluronic F-127. Following fura-II/AM loading, the cells were rinsed 2X with modified testing medium. Intracellular Ca^{2+} imaging was performed within 5 to 60 min of fura-II/AM loading at 18 °C to 20 °C. Changes in $[Ca^{2+}]_i$ were determined by the ratio of the emission intensity of fura-II fluorescence subsequent to excitation at 340 nm and 380 nm (340/380 ratio). Data were expressed as the 340/380 ratio, or as $[Ca^{2+}]_i$ (see chapter 6). Due to variation in basal 340/380 ratio values ($[Ca^{2+}]_i$) between experiments, the data were normalized to the mean value obtained in the first three ratio images and were expressed as % pretreatment. All values in the text and figures are reported as mean ± SEM.

Electrophysiological Recordings

Whole-cell, voltage-clamp recordings (Hamill *et al.*, 1981) were performed at room temperature (18 °C to 20 °C) using nystatin-perforated-patch recording techniques (Korn and Horn, 1989), previously described in Chapter 3. Voltage-dependent Ca^{2+} and K^+ currents were continuously monitored during 40 msec voltage-steps to 0 mV from a holding potential of -70 mV, delivered every 5 sec. Current-voltage relationships were generated only after the response to the drug application had stabilized.

The composition (in mM) of the external solutions for the different recording conditions were as follows:

1. Ba^{2+} currents (through voltage-dependent Ca^{2+} channels): 130 N-methyl-D-glucamine-Cl, 20 $BaCl_2$, 1.0 $MgCl_2$, 2.5 KCl, 8 glucose, 10 HEPES (pH adjusted to 7.2 with HCl).

2. K^+ currents: 142 NaCl, 6.0 $CaCl_2$, 2.0 KCl, 3.0 $MgCl_2$, 0.001 TTX, 10 glucose and 10 HEPES (pH adjusted to 7.2 with TrisCl).

The composition (in mM) of the electrode solutions for the different recording conditions were as follows:

1. Ba^{2+} currents: 70 CsCl, 70 cesium-glutamate, 2 $MgCl_2$, 10 HEPES (pH adjusted to 7.2 with TrisCl).

2. K^+ currents: 120 K-aspartate, 20 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES (pH adjusted to 7.2 with TrisCl).

Data analysis was performed using an AT 486 2DX compatible computer in conjunction with Clampfit (Axon Instruments, Foster City, CA). The junction potentials for K^+ and Ba^{2+} current recordings were calculated with the JPCalc program (Barry 1994) to be less than 3.0 mV and were not corrected. All values in the text are reported as mean ± SEM. Differences between groups were considered to be significant when $P < 0.05$ using paired *t* test.

Drugs

Phorbol 12-myristate 13-acetate (TPA), 4α-phorbol 12, 13 didecanoate (4α-PDD), and dioctanoyl glycerol (DiC8) were purchased from Research Biochemicals Incorporated (Natick, MA) and dissolved in DMSO. Aliquots of concentrated stock solutions were stored at -20 °C and diluted to final concentrations in saline. The final concentration of DMSO was ≤ 0.1%, which has no effect on basal GTH-II secretion or ionic currents.

RESULTS

Effects of TPA and DiC8 on KCl-stimulated GTH-II secretion.

Previous studies have suggested that PKC interacts with voltage-dependent Ca^{2+} channels to mediate sustained GTH-II secretion (Jobin and Chang, 1992b; Chang *et al.*, 1993; Chang *et al.*, 1996). One possibility is that prolonged activation of PKC enhances voltage-dependent Ca^{2+} channel activity, resulting in increased Ca^{2+} entry. To test this hypothesis, the effects of prior treatment with low doses of PKC activators on voltage-dependent Ca^{2+} channel activity were monitored in perifusion studies. To activate voltage-dependent Ca^{2+} channels, a high concentration of KCl was applied to the extracellular medium. As shown in chapters 5 and 6, 30 mM KCl effectively activated voltage-dependent Ca^{2+} channels and increased $[Ca^{2+}]_i$, leading to hormone secretion. In the present experiments, pretreatment with 1 μM DiC8 or 1 nM TPA for 20 min augmented KCl-stimulated GTH-II secretion (Fig. 7.1 and 7.2) However, 1 nM 4α-PDD, an inactive phorbol analog, did not alter KCl-stimulated GTH-II secretion (Fig. 7.2). Application of these relatively low doses of DiC8 or TPA alone caused a relatively small increase in GTH-II secretion compared to the response in the presence of KCl

(Fig. 7.1 and 7.2). These data indicate that prolonged exposure to PKC can enhance GTH-II secretion that is elicited by activation of voltage-dependent Ca^{2+} channels.

Effects of TPA and 4α-PDD on $[Ca^{2+}]_i$.

To further investigate the effects of PKC activation on Ca^{2+} mobilization in goldfish gonadotrophs, single-cell Ca^{2+} imaging experiments were performed. Application of 100 nM TPA increased $[Ca^{2+}]_i$ by 35.7 ± 12.1% (mean ± SEM; range = 12.3 to 71.4%; $P < 0.05$ *vs*. 0 change; one-group *t*-test; n = 5; Fig. 7.3). This corresponds to a net increase in estimated $[Ca^{2+}]_i$ of 134 ± 49 nM (mean ± SEM; range 38 to 168; n = 5). Unlike TPA, 100 nM 4α-PDD did not significantly increase $[Ca^{2+}]_i$ ($P > 0.05$; n = 3; Fig. 7.3). These results suggest that activation of PKC can stimulate an increase in $[Ca^{2+}]_i$ in goldfish gonadotrophs.

In identified goldfish gonadotrophs, GnRH stimulates a localized increase in $[Ca^{2+}]_i$ near one pole of the cell (Chapter 6). A similar localized increase was observed in response to a 2 min application of 100 nM TPA (Figure 7.4). In these cells, the increase in $[Ca^{2+}]_i$ appeared to be greatest near the plasma membrane and progressively smaller towards the center of the cell.

Effects of PKC on voltage-dependent Ba^{2+} and K^+ currents.

The above results demonstrate that activation of PKC stimulates a localized increase in $[Ca^{2+}]_i$. It is possible that PKC increased $[Ca^{2+}]_i$ by enhancing Ca^{2+} influx through voltage-dependent Ca^{2+} channels. To test this hypothesis, the effects of TPA on isolated Ba^{2+} currents were examined using perforated patch-clamp recordings. Barium was used as the charge carrier through voltage-dependent Ca^{2+} channels for the reasons outlined in Chapter

3. Application of 100 (*n* = 4) and 1000 nM (*n* = 4) TPA did not significantly alter the amplitude of voltage-dependent Ba^{2+} currents in identified goldfish gonadotrophs (Table 7.1; Fig. 7.5 A and B).

Activation of PKC may indirectly alter voltage-dependent Ca^{2+} channel activity by altering other ionic currents that influence plasma membrane excitability. To begin to test this hypothesis, the actions of TPA on total outward K^+ currents were examined using perforated-patch-clamp recordings. Outward K^+ currents were elicited by a series of 1 sec voltage-steps from -70 to +80 mV, delivered in 10 mV increments from a holding potential of -80 mV. As in earlier experiments (Chapter 3), the total outward current consisted of a fast-activating transient and sustained lower amplitude component. Application of 100 nM TPA did not alter the peak (0 - 20 msec) or sustained (980 to 1000 msec) outward K^+ current amplitude at any of the test potentials (Fig. 7.6A; *n* = 3). In addition, 100 nM TPA did not alter the current-voltage relation of the outward K^+ current (Fig. 7.6B). These data indicate that activation of PKC does not alter K^+ currents in goldfish gonadotrophs.

DISCUSSION

Based on results from hormone release studies and Ca^{2+} imaging experiments using mixed populations of dispersed pituitary cells, it has been predicted that activation of PKC stimulates an increase in $[Ca^{2+}]_i$ to stimulate GTH-II secretion (Jobin and Chang, 1992b). Using morphologically identified goldfish gonadotrophs, it is now possible to directly monitor the effects of PKC activation on changes in $[Ca^{2+}]_i$. As a result, this study provides the first direct evidence that activation of PKC stimulated an increase in $[Ca^{2+}]_i$ in goldfish gonadotrophs. In addition, activation of PKC stimulates a localized increase in $[Ca^{2+}]_i$, similar to that observed in response to sGnRH and cGnRH-II (Chapter 6). These correlative observations are consistent with the involvement of PKC in mediating GnRH-stimulated GTH-II secretion. However, further studies using inhibitors of PKC are required to more clearly implicate PKC in mediating the GnRH-induced increase in $[Ca^{2+}]_i$.

Activation of PKC may stimulate an increase in $[Ca^{2+}]_i$ by directly activating voltage-dependent Ca^{2+} channels, modulating plasma membrane excitability to indirectly enhance Ca^{2+} entry, or by stimulating and/or modulating Ca^{2+} release from intracellular stores. For example, activation of PKC has been demonstrated to enhance Ca^{2+} currents by the removal of tonic G protein-mediated inhibition in a variety of central and peripheral neurons (Swartz, 1993). Additionally, activation of PKC has been demonstrated to increase Ba^{2+} current amplitude in αT3 gonadotroph cell lines (Bosma and Hille, 1992). In the present study, however, acute activation of PKC did not alter current influx through voltage-dependent Ca^{2+} channels. Additionally, activation of PKC did not alter the amplitude or current-voltage relationship of voltage-dependent K^+ currents. These negative results can't be attributed to an inability to observe agonist-induced changes in current under the present recording conditions. Dopamine D_2 agonists inhibit voltage-dependent Ca^{2+} currents in these cells (Chapter 5), whereas cAMP analogs enhance Ca^{2+} currents (Appendix 3). Together, these data indicate that PKC does not directly activate voltage-dependent Ca^{2+} channels or modulate K^+ channel activity to stimulate an increase in $[Ca^{2+}]_i$. Therefore, it is likely that acute PKC activation mobilizes Ca^{2+} from intracellular stores to increase $[Ca^{2+}]_i$. In support of this view, activation of PKC by DiC8 increases $[Ca^{2+}]_i$ in mixed populations of dispersed goldfish gonadotrophs in part by the mobilization of intracellular Ca^{2+} (Jobin and Chang, 1992b). PKC may act directly on $InsP_3$-

gated channels to modulate the release of Ca^{2+} or it may act on PLC enzymes to increase $InsP_3$ production. Alternatively, PKC may reduce thapsigargin-sensitive Ca^{2+} pump activity that normally counteracts the constant leak of Ca^{2+} from $InsP_3$-gated Ca^{2+} channels. Further studies are required to elucidate the site or sites of action of PKC in the mediation of intracellular Ca^{2+} mobilization.

Although results from the present investigation indicate that acute activation of PKC does not regulate voltage-dependent Ca^{2+} channel activity, they do not negate the possibility that long-term PKC action requires Ca^{2+} entry. In static incubation studies, PKC-stimulated GTH-II secretion is reduced by the removal of extracellular Ca^{2+} or by the addition of voltage-dependent Ca^{2+} channel blockers (Chang *et al.*, 1991a; Jobin and Chang, 1992b). Conversely, Bay K 8644 and membrane depolarization by high concentrations of extracellular K^+ augment PKC-stimulated GTH-II secretion (Jobin and Chang, 1992b; Jobin *et al.*, 1996). These studies indicate that the long-term action of PKC requires Ca^{2+} entry through voltage-dependent Ca^{2+} channels. It is likely that Ca^{2+} entry through voltage-dependent Ca^{2+} channels is required for maintaining intracellular Ca^{2+} stores during prolonged stimulation by PKC (see Chapter 8).

Despite the lack of evidence for direct action of PKC on voltage-dependent Ca^{2+} channels, results from the present study indicate that prolonged exposure to PKC augments the GTH-II response to voltage-dependent Ca^{2+} channel activation. Similarly, activation of PKC enhanced the GTH-II response to KCl in static incubation experiments (Jobin *et al.*, 1996). There are at least two possible explanations for these results. Firstly, PKC may enhance the activity of voltage-dependent Ca^{2+} channels. However, this seems unlikely as PKC does not appear to directly alter voltage-dependent Ca^{2+}

channel activity in these cells. Secondly, PKC may sensitize the exocytotic machinery resulting in enhanced GTH-II secretion. In support of this view, the Ca^{2+} ionophore ionomycin was unable to stimulate GTH-II secretion in dispersed goldfish pituitary cells depleted of PKC (Jobin *et al.*, 1993). This suggests that at least a basal level of PKC is required for exocytosis in goldfish gonadotrophs. Similarly, it has been proposed that PKC sensitizes Ca^{2+} dependent exocytotic mechanisms to support secretion at physiological concentrations of $[Ca^{2+}]_i$ in rat gonadotrophs (Jobin *et al.*, 1995). Moreover, PKC has been demonstrated to stimulate exocytosis in the virtual absence of Ca^{2+} in permeabilized sheep gonadotrophs (Van Der Merwe *et al.*, 1989).

In summary (Fig. 7.7), results from the preliminary studies reported in this chapter indicate that the acute activation of PKC stimulates a local increase in $[Ca^{2+}]_i$, which does not appear to be mediated by direct actions on voltage-dependent Ca^{2+} channel activity. However, these studies support the hypothesis that PKC sensitizes the exocytotic machinery to facilitate Ca^{2+}-dependent GTH-II secretion.

Table 7.1 Effects of TPA on voltage-dependent Ba^{2+} current in identified goldfish gonadotrophs.

	Peak Ba^{2+} current amplitude (pA)[a]	
Treatment	Control	TPA
100 nM TPA (*n* = 4)	81.1 ± 26.4	88.1 ± 29.7
1000 nM TPA (*n* = 4)	176.6 ± 76.2	186.5 ± 68.2

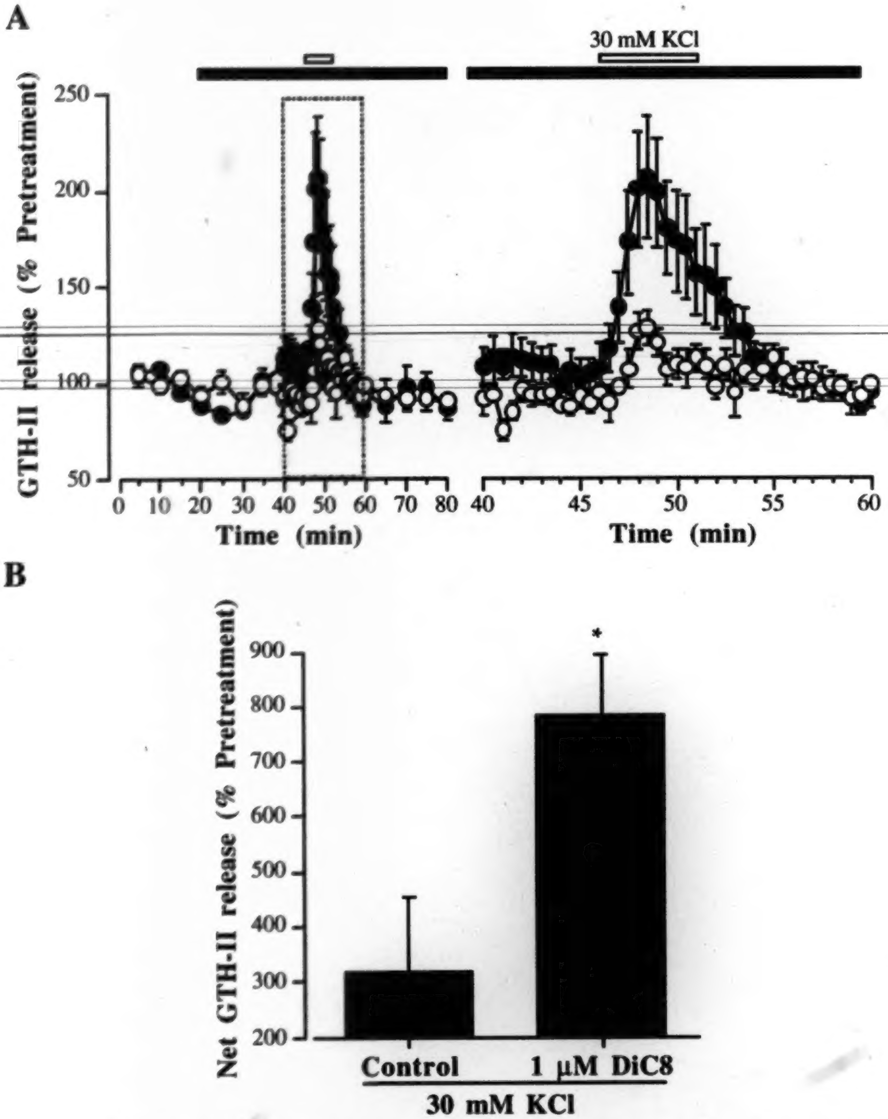


Fig. 7.1. Effects of DiC8 on K^+-stimulated GTH-II secretion from dispersed pituitary cells under perifusion conditions. A. Left panel: GTH-II release response to a 5 min pulse of 30 mM KCl (*open bar*) in the presence (*filled bar*) of DMSO (*open circles*) or 1 μM DiC8 (*filled circles*). Right panel: expanded view of the boxed area in the left panel. B. Net GTH-II release response to 30 mM K^+ in the presence of DMSO or 1 μM DiC8 (n = 4 and 6, respectively). Responses (mean ± SEM) were normalized as a percentage of the pretreatment values (9.31 ± 1.85 ng/ml/2 x 10^6 cells). *Asterisk* denotes significant difference from control (unpaired t-test, $P < 0.05$).

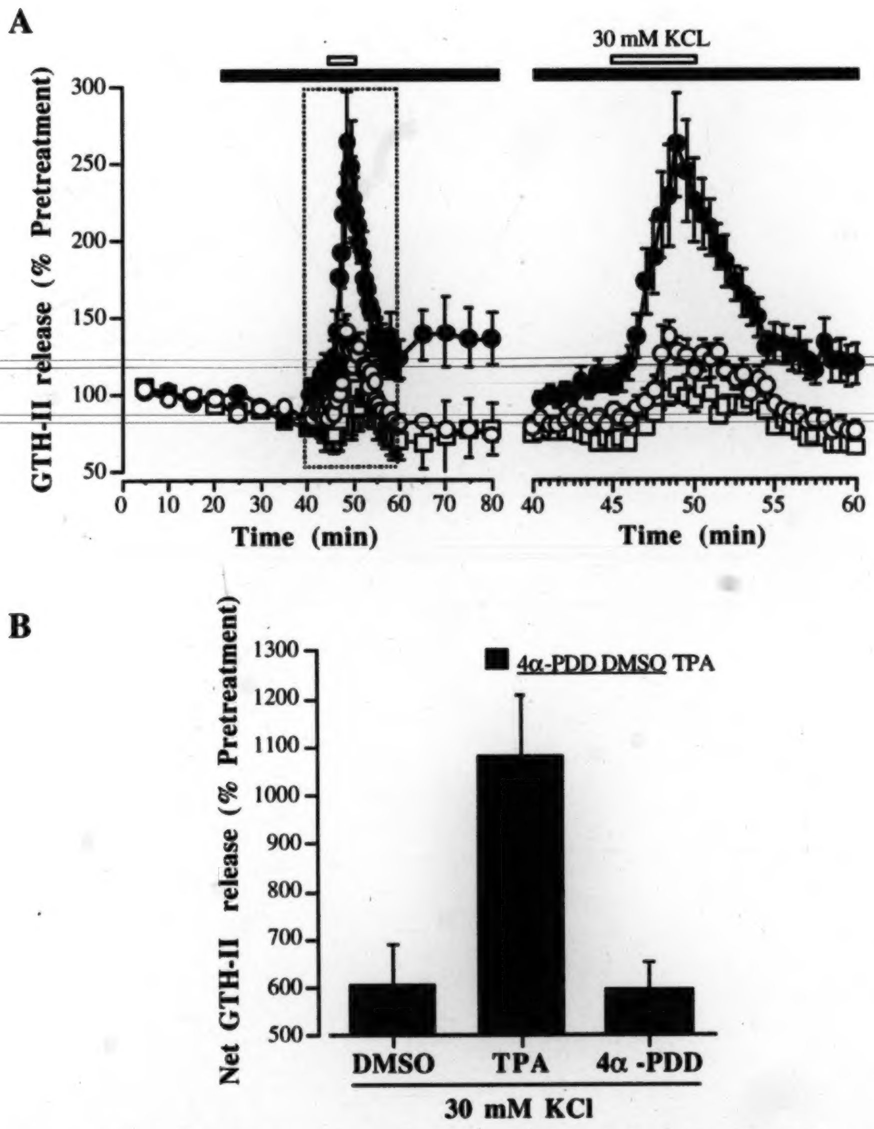


Fig. 7.2. Effects of TPA and 4α-PDD on K^+-stimulated GTH-II secretion from dispersed pituitary cells under perifusion conditions. A. Left panel: GTH-II release response to a 5-min pulse of 30 mM KCl (*open bar*) in the presence (*filled bar*) of DMSO (*open circles*), 1 nM TPA (*filled circles*) or 1 nM 4α-PDD (*open squares*). Right panel: expanded view of the boxed area in the left panel. B. Net GTH-II release response to 30 mM K^+ in the presence of DMSO, 1 nM TPA or 1 nM 4α-PDD ($n = 6$ to 8). Responses (mean ± SEM) were normalized as a percentage of the pretreatment values (9.66 ± 0.48 ng/ml/2 x 10^6 cells). Treatment groups having similar GTH-II responses (ANOVA followed by Fisher's LSD test, $P > 0.05$) are identified by the same underscore.

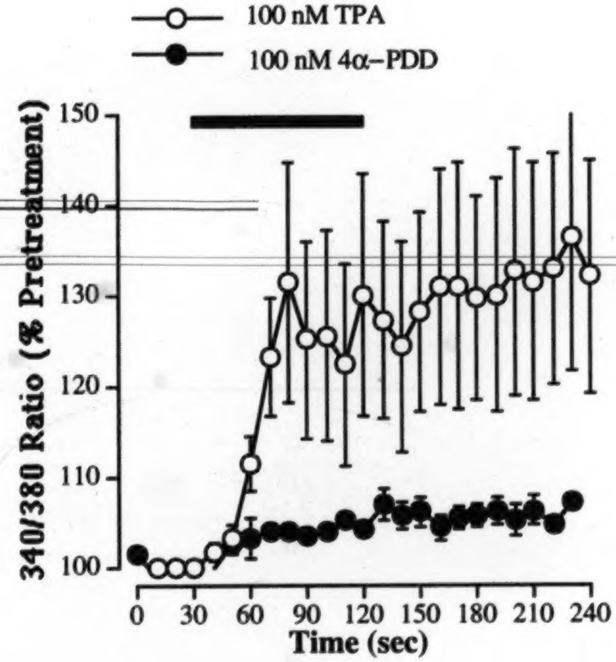


Fig. 7.3. Effects of TPA and 4α-PDD on $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. $[Ca^{2+}]_i$ responses (mean ± SEM) to a 2 min application (*bar*) of 100 nM TPA ($n = 5$; *open circles*) or 100 nM 4α-PDD ($n = 3$; *filled circles*) are shown. Results from separate experiments using gonadotrophs prepared from prespawning and regressed goldfish were normalized as a percentage of pretreatment 340/380 ratio values (TPA: 1.10 ± 0.04; 4α-PDD: 1.38 ± 0.09).

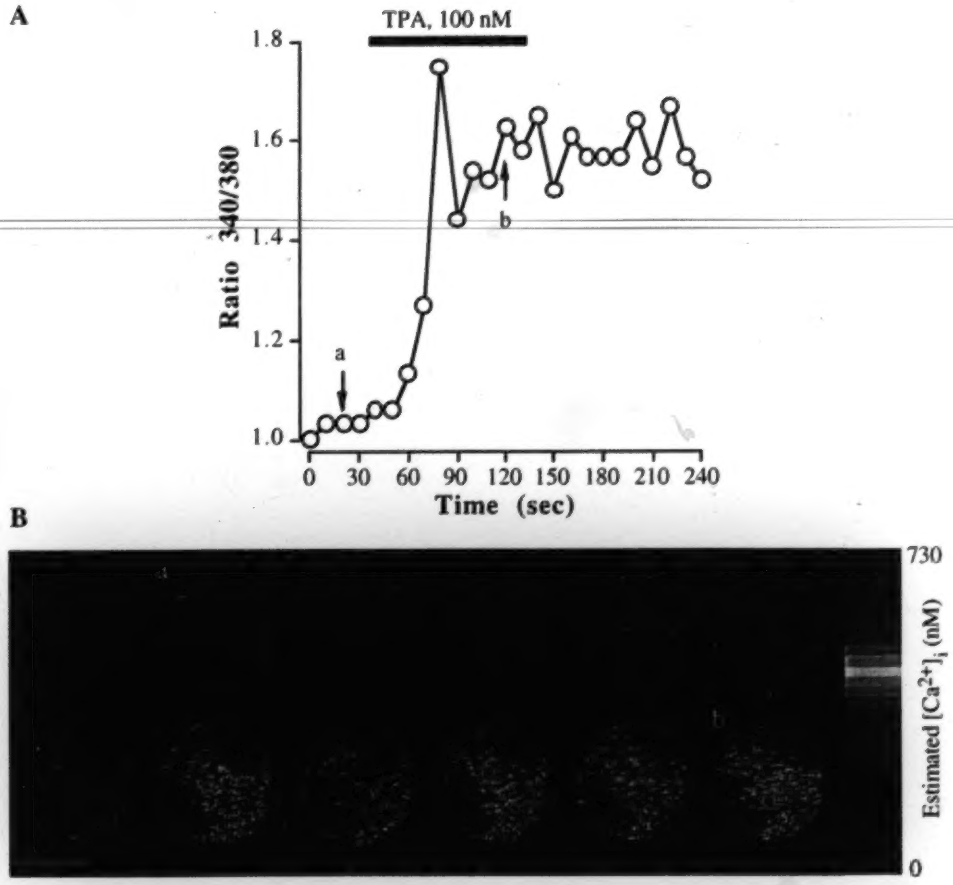


Fig. 7.4. TPA-induced changes in $[Ca^{2+}]_i$ in identified goldfish gonadotrophs. A. Temporal pattern of 100 nM TPA-induced (*bar*) changes in 340/380 ratio. Ratio values were collected once every 10 sec. B. A series of pseudo-color ratio images at the times indicated in (A) showing the localized response to TPA. Bar: 10 µm.

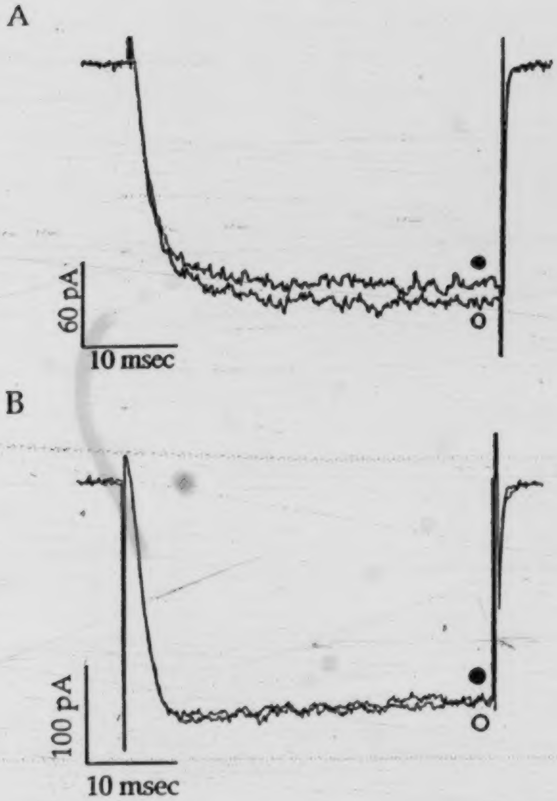


Fig. 7.5. Effects of TPA on voltage-dependent Ba^{2+} currents in identified goldfish gonadotrophs. Representative superimposed Ba^{2+} current traces in the presence (*filled circle*) and absence (*open circle*) of 100 nM (A) or 1000 nM (B) TPA are shown. All currents were elicited during a 40 msec voltage step to +10 mV from a holding potential of -80 mV. Experiments were performed with gonadotrophs prepared from sexually recrudescent and prespawning goldfish.

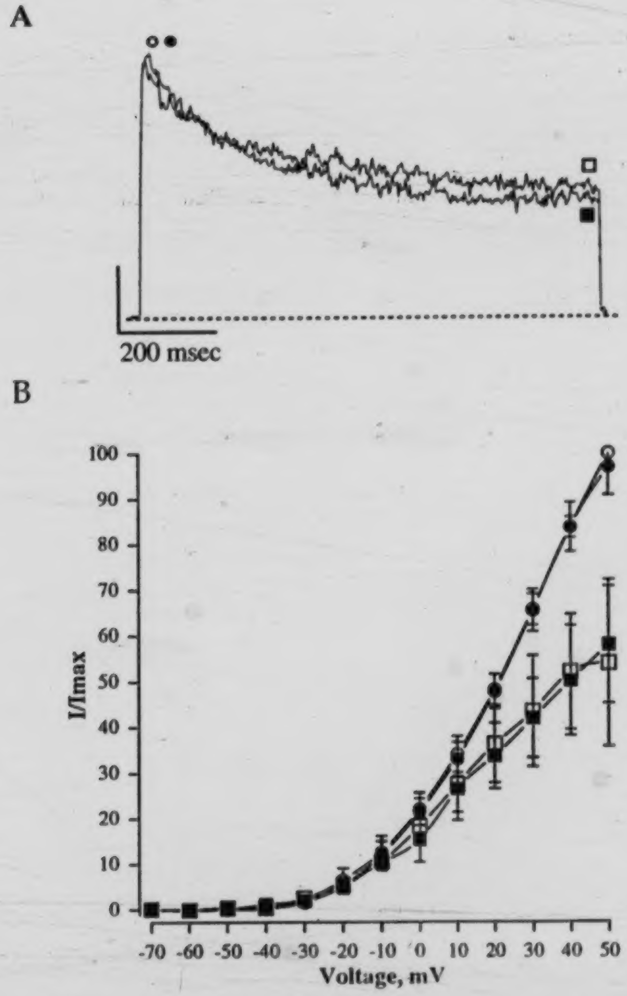


Fig. 7.6. Effect of TPA on voltage-dependent K+ current in identified goldfish gonadotrophs. A. Superimposed K+ currents in the presence (*open sympols*) or absence (*filled symbols*) of 100 nM TPA. Currents were elicited by a 1000-msec voltage-step to +10 mV from a holding potential of -80 mV. B. Current-voltage relationship of the peak (*circles*) and steady-state (*squares*) K+ current elicited by 1000 msec voltage-steps to the potentials indicated (holding potential = -80 mV) in the presence (*filled symbols*) or absence (*open symbols*) of 100 nM TPA. All currents were normalized to the maximal inward current observed in the absence of TPA, and are presented as mean ± SEM ($n = 3$).

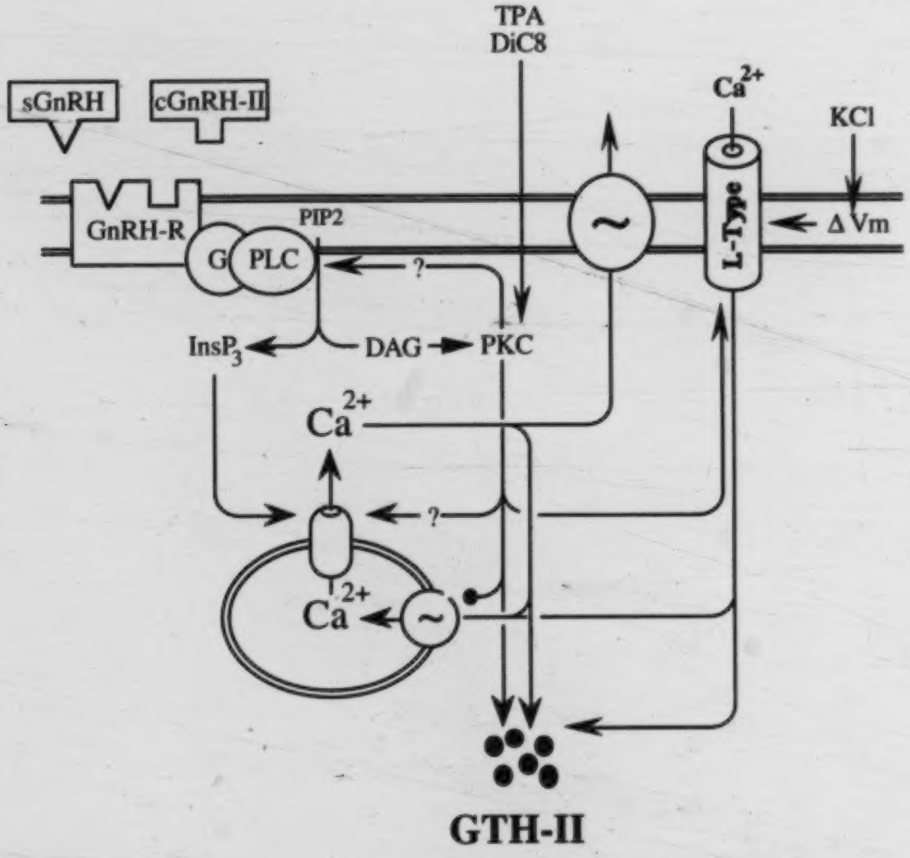


Fig. 7.7. Actions of PKC on intracellular and extracellular Ca^{2+} mobilization in goldfish gonadotrophs. Abbreviations include: TG = thapsigargin, R = receptor, G = G-protein, PLC = phospholipase C, PIP = phosphatidylinositol-bisphosphate ($PI[4, 5]P_2$), InsP3 = Inositol (1, 4, 5)-trisphosphate, ~ = Ca^{2+} pump.

Chapter 8

GENERAL DISCUSSION

Previously, the involvement of intracellular Ca^{2+} stores and voltage-dependent ionic channels in the regulation of GnRH and dopamine action in fish gonadotrophs has been based primarily on indirect studies using pituitary fragments or mixed populations of dispersed pituitary cells. The development of the cell identification protocol in Chapter 2 allowed the electrical membrane activity and $[Ca^{2+}]_i$ response to GnRH and dopamine to be directly monitored in single, identified goldfish gonadotrophs.

These studies led to several significant developments in our understanding of gonadotroph cell function in goldfish: (1) the electrical membrane properties and voltage-dependent ionic currents of identified goldfish gonadotrophs were characterized with standard- and perforated-patch whole-cell patch-clamp recordings; (2) the importance of extracellular Na^+ and Na^+/H^+ exchange in the regulation of GnRH action under physiological conditions was demonstrated; (3) inhibition of voltage-dependent Ca^{2+} channels by dopamine D_2 receptor activation was demonstrated to be an important mechanism mediating dopamine action; (4) a single-cell Ca^{2+} imaging protocol using fura-II/AM was set up and verified for identified goldfish gonadotrophs; (5) preliminary fura-II Ca^{2+} imaging experiments demonstrated that GnRH and PKC stimulate a localized increase in $[Ca^{2+}]i$, which is in part due to the mobilization of $[Ca^{2+}]_i$ from intracellular stores; (6) fura-II studies indicated that both sGnRH and cGnRH-II can act on the same cell to stimulate an increase in $[Ca^{2+}]_i$. These single-cell studies are important for understanding how individual gonadotrophs contribute to the GnRH- and dopamine-induced responses observed in studies of mixed-cell populations.

The ability to directly identify individual goldfish gonadotrophs, somatotrophs and lactotrophs represents a significant advancement in the study of neuroendocrine action in teleosts (Chapter 2). The cells identified in the present study by their morphological characteristics represent a subpopulation of their respective cell types. In future studies, it should be possible to identify other subpopulations of these cells or other hormone secreting cell types in dispersed goldfish pituitary cell cultures. There are at least two other cell types which exhibit easily recognizable and unique cellular morphologies (F. Van Goor, unpublished observations). It has yet to be determined if they represent other subpopulations of a cell-type already identified, or if they represent other hormone secreting cell-types, such as thyrotrophs, corticotrophs, melanotrophs, somatolactotrophs or GTH-I containing cells. The use of cell-type specific morphologies may be applicable to the identification of pituitary cells in other vertebrate species. For example, although the technique to identify cells based on their unique cellular morphologies has not been formally described or validated, at least one other group uses cell type specific morphology to identify gonadotrophs from normal and ovariectomized rats (Tomic *et al.*, 1994).

Earlier studies have suggested that sGnRH and cGnRH-II activate PKC, which in turn stimulates Ca^{2+} entry through dihydropyridine-sensitive Ca^{2+} channels, resulting in GTH-II secretion. Preliminary studies suggest that dopamine inhibits GnRH-induced Ca^{2+} entry to inhibit GTH-II secretion (reviewed in Chapter 1). These studies lead to the hypothesis that GnRH and dopamine act on voltage-dependent Ca^{2+} channels to regulate $[Ca^{2+}]_i$ and

GTH-II secretion. This was directly tested in the present study using patch-clamp recording and single-cell Ca^{2+} imaging techniques performed on single identified goldfish gonadotrophs. Results from these experiments were often accompanied by parallel hormone release experiments using cell-column perifusion with the collection of 30 sec and 5 min fractions, as well as static incubation. This allowed the activity of single, identified cells to be related to the short-term and prolonged GTH-II-release response observed in pituitary cell populations.

Several steps were taken to demonstrate the presence of dihydropyridine-sensitive Ca^{2+} channels in identified goldfish gonadotrophs. High concentrations of extracellular KCl stimulated GTH-II secretion in an extracellular Ca^{2+}- and nifedipine-dependent manner (Chapter 5). Bay K 8644 stimulated GTH-II secretion (Chapter 4 and 5). High concentrations of extracellular KCl and Bay K 8644 stimulated an increase in $[Ca^{2+}]_i$ in identified goldfish gonadotrophs (Chapter 6). Tight-seal- and perforated-patch-clamp recordings directly demonstrated the presence of high-voltage activated, dihydropyridine-sensitive Ca^{2+} channels in identified goldfish gonadotrophs (Chapter 3). Together, these data confirm the presence of dihydropyridine-sensitive Ca^{2+} channels in identified goldfish gonadotrophs, indicating that they may be a possible target for the regulation of GTH-II secretion by GnRH and dopamine.

Further studies identified voltage-dependent Ca^{2+} channels as a target for dopamine action in identified goldfish gonadotrophs. Activation of dopamine D_2 receptors reduced KCl- and Bay K 8644-stimulated GTH-II secretion. Dopamine D_2 receptor activation reduced voltage-dependent Ba^{2+} current amplitude in identified goldfish gonadotrophs (Chapter 5). Dopamine may alter the activation and inactivation kinetics of the current to inhibit Ca^{2+} channels (Chapter 5). Dopamine may also remove a fast-activating, slowly inactivating current subtype, which is insensitive to dihydropyridines. To determine whether dopamine acts only on the dihydropyridine-sensitive component of this current or inhibits the activity of additional Ca^{2+} channel subtypes which may be present, requires further testing. Nevertheless, the inhibitory action of dopamine on voltage-dependent Ca^{2+} channels is consistent with the hypothesis that these channels are involved in the neuroendocrine regulation of GTH-II secretion.

The hypothesis that sGnRH and cGnRH-II activate PKC to stimulate Ca^{2+} entry through voltage-dependent Ca^{2+} channels was tested in single-cell Ca^{2+} imaging and electrophysiological experiments. These experiments indicated that sGnRH, cGnRH-II and activation of PKC stimulated an increase in $[Ca^{2+}]_i$ (Chapter 6). Although GnRH and Bay K 8644 stimulated increases in $[Ca^{2+}]_i$ in the same region of the cell, there was no clear evidence that Ca^{2+} entry through voltage-dependent Ca^{2+} channels participated in the response to GnRH. Moreover, sGnRH, cGnRH-II or PKC activators did not affect voltage-dependent Ba^{2+} currents in identified goldfish gonadotrophs (Chapter 6 and 7). These data do not support previous studies (reviewed in Chapter 1) which suggest that activation of PKC by GnRH directly stimulates Ca^{2+} entry through voltage-dependent Ca^{2+} channels.

Several explanations may account for the apparent inconsistencies observed between this study and other studies. It is possible that the morphologically identified gonadotrophs used in this study represent a subpopulation of cells which do not activate voltage-dependent Ca^{2+} channels to stimulate GTH-II secretion. As well, the subpopulation of gonadotrophs may not secrete GTH-II. In hormone release studies, prolonged inhibition of extracellular Ca^{2+} was suggested to be responsible for inhibiting

GnRH-stimulated GTH-II secretion (Jobin *et al.*, 1996); however, this have also lead to the depletion of intracellular Ca^{2+} stores. An alternative explanation for these results may be that sustained Ca^{2+} entry is required to maintain GnRH-sensitive intracellular Ca^{2+} stores. Future testing is required to resolve these inconsistencies.

PROPOSED MODEL OF GNRH AND DOPAMINE ACTION IN GOLDFISH GONADOTROPHS.

Unstimulated goldfish gonadotrophs.

In many excitable neuronal and endocrine cells, basal $[Ca^{2+}]_i$ is tightly regulated due to its important role in stimulus-secretion coupling (Berridge, 1993; Hille *et al.*, 1994, 1995; Stojilkovic and Catt, 1992, 1995; Stojilkovic *et al.*, 1994). The maintenance of low, non-fluctuating levels of $[Ca^{2+}]_i$ and the rapid return to basal $[Ca^{2+}]_i$ following ionomycin treatment, demonstrate that goldfish gonadotrophs can effectively regulate their $[Ca^{2+}]_i$ (Chapter 6). In general, basal $[Ca^{2+}]_i$ can be influenced by Ca^{2+} influx or efflux across the plasma membrane and Ca^{2+} release or sequestration by the intracellular Ca^{2+} stores. In this study, it was demonstrated that both Ca^{2+} entry and sequestration are involved in the regulation of basal $[Ca^{2+}]_i$. Several lines of evidence indicate that Ca^{2+} entry through voltage-dependent Ca^{2+} channels may occur during the spontaneous action potentials which were observed in some gonadotrophs (Chapter 3). The action potentials are only partially sensitive to concentrations of TTX which abolished voltage-dependent Na^+ currents, suggesting that voltage-dependent Ca^{2+} channels also contribute to the action potential (Chapter 3). At resting membrane potential, > 80% of the voltage-dependent Ca^{2+} channels are available for activation (Chapter 3).

Application of Bay K 8644, which maintains voltage-dependent Ca^{2+} channels in the open state, increased $[Ca^{2+}]_i$ in identified goldfish gonadotrophs (Chapter 6) and stimulated GTH-II secretion under static incubation conditions (Chapter 4 and 5). Recent studies have also demonstrated that inhibition of the I_A-type K^+ current stimulated an increase in basal GTH-II secretion (J. P. Chang, unpublished observations). This is likely due to action potential spike-broadening and the resulting increase in Ca^{2+} entry. It thus appears that $[Ca^{2+}]_i$ in unstimulated goldfish gonadotrophs is regulated, at least in part, by the activity of both voltage-dependent Ca^{2+} channels and endoplasmic reticulum Ca^{2+}-ATPase.

In addition to Ca^{2+} entry during spontaneous action potentials, Ca^{2+} sequestration by intracellular Ca^{2+} stores also appears to regulate $[Ca^{2+}]_i$. Treatment with thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, stimulated a rapid increase in $[Ca^{2+}]_i$ in identified goldfish gonadotrophs (Chapter 6). Thapsigargin also stimulated GTH-II secretion in static incubation experiments (J. P. Chang and R. Garofalo, unpublished observations). This indicates that intracellular Ca^{2+} stores spontaneously release Ca^{2+}, which is continuously re-sequestered by a thapsigargin-sensitive Ca^{2+}-ATPase. Alternatively, thapsigargin-sensitive stores may be located close to the plasma membrane, where they sequester Ca^{2+} which enters during spontaneous action potential activity. The appearance of Ca^{2+} 'hot spots' near to the plasma membrane during thapsigargin treatment is consistent with this hypothesis.

In addition to regulating $[Ca^{2+}]_i$, the activity of the plasma membrane also appears to be important in determining the concentration of Ca^{2+} in the intracellular Ca^{2+} stores. In the presence of thapsigargin, a decrease in Ca^{2+} entry through voltage-dependent Ca^{2+} channels can, in some cases, stimulate an increase in $[Ca^{2+}]_i$ (Chapter 6). These results are consistent with previous studies which demonstrated that removal of extracellular Ca^{2+}, as well as the addition of inorganic and organic Ca^{2+} channel antagonists, stimulated a transient increase in GTH-II secretion (Jobin, 1993). As these treatments impeded Ca^{2+} entry through voltage-dependent Ca^{2+} channels to varying degrees (Chapter 3), it is likely that the increase in $[Ca^{2+}]_i$ is due to a stimulation of Ca^{2+} release, or an inhibition of Ca^{2+} sequestration by the intracellular stores. This suggests that a decrease in Ca^{2+} influx directly or indirectly alters the activity of the intracellular Ca^{2+} stores. It can also be hypothesized that during prolonged inhibition of Ca^{2+} entry the concentration of Ca^{2+} within the intracellular Ca^{2+} stores would be reduced. Conversely, it has been suggested that enhanced Ca^{2+} entry can increase the concentration of Ca^{2+} in the intracellular Ca^{2+} store (Jobin, 1993). This is suggested by perifusion studies in which dispersed goldfish pituitary cells were exposed to normal or high concentrations of extracellular Ca^{2+} prior to the removal of extracellular Ca^{2+}. In the presence of high concentrations of extracellular Ca^{2+}, which would increase the concentration gradient across the plasma membrane resulting in increased Ca^{2+} entry, GTH-II secretion was reduced; however, the GTH-II release response to the removal of extracellular Ca^{2+} was enhanced (Jobin, 1993). If this response is due to intracellular Ca^{2+} release, then these observations suggest that enhanced Ca^{2+} entry increases the concentration of Ca^{2+} in the intracellular Ca^{2+} stores. Taken together, these data are consistent with the hypothesis that the degree of Ca^{2+} entry through voltage dependent Ca^{2+} channels can determine the concentration of Ca^{2+} in the intracellular Ca^{2+} stores.

GnRH action in goldfish gonadotrophs.

In goldfish, it has been suggested that sGnRH and cGnRH-II bind to the same population of GnRH receptors to stimulate an increase in $[Ca^{2+}]_i$, which in turn leads to GTH-II secretion (Jobin and Chang, 1992a, 1992b). Consistent with this hypothesis, acute activation of GnRH receptors stimulated an increase in $[Ca^{2+}]_i$ in a subpopulation of goldfish gonadotrophs (Chapter 6). This increase in $[Ca^{2+}]_i$ is primarily due to the mobilization of intracellular Ca^{2+}, as inhibition of Ca^{2+} entry did not significantly reduce the response to GnRH (Chapter 6). An increase in $InsP_3$ levels, via a G-protein/PLC-dependent second messenger pathway, may stimulate the release of intracellular Ca^{2+}; however, there may be a difference in the metabolic pathway of $InsP_3$ following GnRH-receptor activation by sGnRH as compared to cGnRH-II. Salmon-GnRH increased $InsP_3$ and higher InsPs levels by 10 min, but no such increase was observed following cGnRH-II treatment. In contrast, an increase in $InsP_2$ was observed following 10 min of stimulation by both sGnRH and cGnRH-II (Chang *et al.*, 1995). It is possible that while GnRH receptor activation by sGnRH may lead to a sustained increase in $InsP_3$, cGnRH-II may stimulate transient or low level increases. The sustained production of higher InsPs may also lead to further differences in the postreceptor second messenger pathways between the two GnRHs. Further studies will be required to determine more accurately the time course of $InsP_3$ production to elucidate its role in mediating the actions of both native GnRHs.

Activation of PLC by sGnRH and cGnRH-II also has been linked to the production of DAG and the subsequent activation of PKC in goldfish gonadotrophs (Chang *et al.*, 1993; Chang and Jobin, 1994; Chang *et al.*, 1996). In the present study, activation of PKC stimulated a localized increase in

$[Ca^{2+}]_i$ within the same region(s) of the cell as GnRH (Chapter 7). This suggests that the activation of PKC by GnRH may stimulate Ca^{2+} release from intracellular Ca^{2+} stores; however, PKC may also act at other points along the cellular signaling cascade to mediate the actions of sGnRH and cGnRH-II. For example, activation of PKC may be required to maintain dihydropyridine-sensitive Ca^{2+} channel activity during long-term exposure to GnRH (reviewed in Chapter 1). In addition, PKC may activate amiloride-sensitive Na^+/H^+ antiports to offset acidification resulting from GnRH-induced increases in $[Ca^{2+}]_i$ (Chapter 4), thus maintaining optimal conditions for secretion. Activation of PKC may also have direct actions on the exocytotic machinery to enhance Ca^{2+}-activated GTH-II secretion. In support of this view, the Ca^{2+} ionophore, ionomycin, was unable to stimulate GTH-II secretion in PKC-depleted cells (Jobin *et al.*, 1993). Activation of PKC augments K^+-stimulated GTH-II secretion (Chapter 7), further suggesting that PKC enhances Ca^{2+}-dependent exocytosis in goldfish gonadotrophs.

Dopamine action in goldfish gonadotrophs.

In goldfish, dopamine exerts tonic inhibitory control over GTH-II secretion throughout the reproductive gonadal cycle, allowing for the maintenance of relatively low circulating GTH-II levels (Peter *et al.*, 1986; Omeljaniuk *et al.*, 1989; Peter *et al.*, 1991). Dopamine or its agonists also inhibit basal GTH-II secretion from goldfish pituitary fragments and dispersed pituitary cells (Chang *et al.*, 1990; Peter *et al.*, 1991). In the pituitary, dopamine acts through dopamine D_2 receptors on GnRH nerve terminals and gonadotrophs to inhibit GTH-II secretion (Peter *et al.*, 1986; Chang *et al.*, 1990b). In the present study, activation of dopamine D_2 receptors reduced voltage-dependent Ba^{2+} current amplitude and voltage-dependent Ca^{2+}

channel-mediated GTH-II secretion (Chapter 5). As discussed above, continuous Ca^{2+} entry during spontaneous action potential activity is believed to be important in maintaining intracellular Ca^{2+} stores; therefore, prolonged inhibition of Ca^{2+} entry due to dopamine-induced inhibition of voltage-dependent Ca^{2+} channels may reduce intracellular Ca^{2+} stores. As a result, the $[Ca^{2+}]_i$ responses to subsequent activation of GnRH receptors would be reduced or inhibited, thus preventing GTH-II secretion.

Removal of dopamine inhibition may also play an important role in priming the intracellular Ca^{2+} stores in preparation for the GnRH-induced GTH-II surge in prespawning goldfish. In in vivo studies, injections of pimozide, a dopamine agonist, potentiated the GTH-II release response to subsequent injections of GnRH in prespawning female goldfish (Chang *et al.*, 1984; Peter *et al.*, 1986, 1992). Treatment with pimozide prior to GnRH injection was more effective in inducing ovulation than the simultaneous application of GnRH and pimozide. These results suggest that prior removal of inhibitory dopamine tone potentiates the response to GnRH. One possible explanation for these observations is that removal of dopamine-induced inhibition of Ca^{2+} channels allows for the optimal filling of intracellular Ca^{2+} stores. As discussed in an earlier section, prior filling of intracellular Ca^{2+} stores increased the subsequent GTH-II release-response (Jobin, 1993); therefore, removal of dopamine-induced inhibition of voltage-dependent Ca^{2+} channels would increase Ca^{2+} entry, resulting in an increase in Ca^{2+} concentration within the intracellular store(s). This priming effect may allow for maximal stimulation of GTH-II secretion by GnRH.

Summary

The development of the cell identification technique outlined in this thesis provided an excellent opportunity to study the physiology of goldfish gonadotrophs with far greater resolution than has been previously possible. In addition, the ability to identify single somatotrophs as well as lactotrophs will surely enhance our knowledge of GH and PRL secretion in the near future. Results from electrophysiological experiments performed on identified goldfish gonadotrophs demonstrated for the first time that these cells are electrically excitable and contain multiple voltage-dependent ionic channels. The combination of hormone release studies and electrophysiological techniques illustrated the importance of extracellular Na^+ in mediating GnRH-stimulated GTH-II secretion. Moreover, the involvement of amiloride-sensitive Na^+/H^+ exchangers in the regulation of cellular pH in goldfish gonadotrophs was demonstrated. It was also demonstrated that dopamine reduced Ca^{2+} channel activity through D_2-type receptors. Results from electrophysiological studies and single-cell Ca^{2+}-imaging studies suggest that continued Ca^{2+} influx is important in determining the availability of intracellular Ca^{2+} in response to GnRH receptor activation by either sGnRH or cGnRH-II. These results, in combination with the inhibitory actions of dopamine on Ca^{2+} entry, lead to the hypothesis that removal of inhibitory dopamine tone allows for increased Ca^{2+} entry through voltage-dependent Ca^{2+} channels, which primes the intracellular Ca^{2+} stores for subsequent stimulation by GnRH. The priming of intracellular Ca^{2+} stores may play an important role in allowing the GTH-II surge to occur, which is a prerequisite for final oocyte maturation in female goldfish.

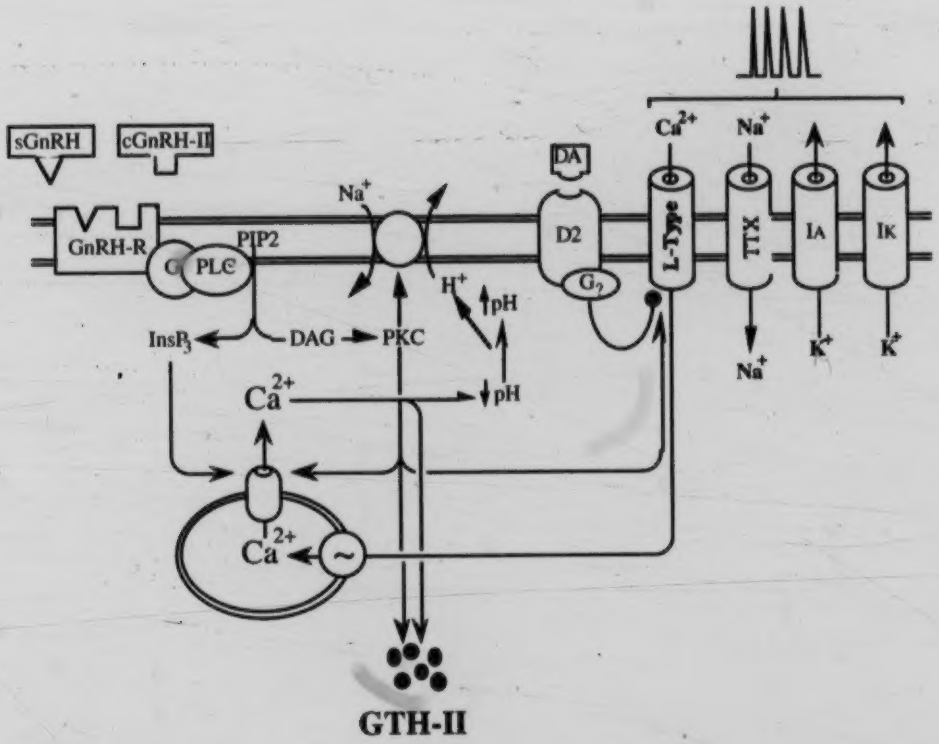


Fig. 8.1. Updated model of GnRH and dopamine signaling in goldfish gonadotrophs.

Chapter 9

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Appendix 1

VOLTAGE-DEPENDENT SODIUM AND CALCIUM CURRENTS IN IDENTIFIED GOLDFISH SOMATOTROPHS[†]

INTRODUCTION

In goldfish, GH secretion is under the stimulatory control of both GnRH and dopamine (Peter *et al.*, 1991; Wong *et al.*, 1993, Chang *et al.*, 1993). The two native GnRHs, sGnRH and cGnRH-II, stimulate GH secretion by activating GnRH receptors located on the membrane of goldfish somatotrophs (Cook *et al.*, 1991). On the other hand, dopamine stimulates GH secretion by the activation of dopamine D1-receptors. Pharmacological studies have demonstrated that voltage-dependent Ca^{2+} channels participate in both GnRH- and dopamine- stimulated GH secretion from the goldfish pituitary. The GH responses to GnRH and dopamine are reduced by the removal of extracellular Ca^{2+}, or the addition of inorganic and organic voltage-dependent Ca^{2+} channel inhibitors (Wong *et al.*, 1994a; Chang *et al.*, 1996). In contrast, the responses are potentiated by the voltage-dependent Ca^{2+} channel agonist, Bay K 8644 (Wong *et al.*, 1994b; Chang *et al.*, 1996). Although these studies indirectly demonstrate the presence of voltage-dependent Ca^{2+} channels in goldfish somatotrophs, these channels and other ionic channels have not been characterized in any teleostean somatotrophs. In this preliminary study, perforated-patch-clamp recording techniques were used to characterize the voltage-dependent Ca^{2+} and Na^+ currents present in morphologically identified goldfish somatotrophs.

[†] Portions of this chapter have been incorporated in Chang *et al.*, 1994. Chin. J. Physiol. 37, 111-127 or Van Goor *et al.*, J. Neuroendocrinol. (submitted July 23, 1996).

MATERIALS AND METHODS

Male and female goldfish pituitaries were excised and their pituitary cells were dispersed and cultured as previously described (Chapter 5). For electrophysiological studies, somatotrophs were identified according to their unique cellular morphology (Chapter 2). Nystatin-perforated patch (Korn and Horn, 1989) recording techniques were used as previously described (Chapter 3). For recordings of inward currents, the pipette was filled with (in mM) 70 Cs-glutamate, 70 CsCl, 2 $MgCl_2$, 10 HEPES and 160 µg/ml nystatin (pH 7.2). For recordings of isolated Na^+ currents, the external bath solution contained (in mM): 136 NaCl, 5 $CaCl_2$, 2.5 KCl, 1 $MgCl_2$, 0.5 $CdCl_2$, 10 TEA-Cl, 8 glucose, and 10 HEPES (pH adjusted to 7.2 with NaOH). For recordings of Ba^{2+} currents through voltage-dependent Ca^{2+} channels, the external bath solution contained (in mM): 120 N-methyl-D-glucamine-Cl, 20 $BaCl_2$, 1.0 $MgCl_2$, 2.5 KCl, 8 glucose and 10 HEPES (pH adjusted to 7.2 with HCl). All reported membrane potentials for Na^+ currents were corrected for a liquid junction potential of 10 mV. The membrane potential for Ba^{2+} currents were not corrected for the liquid-junction potential of 3 mV. ED_{50} values were estimated using a four-parameter logistic curve-fitting program (ALLFIT; DeLean *et al.*, 1978).

RESULTS

Voltage-dependent Na^+ currents

In identified goldfish somatotrophs, a fast-activating and fast-inactivating Na^+ current was detected at command potentials more depolarized than -50 mV (Fig. A.1.1A). The peak inward Na^+ current averaged -82 ± 15 pA (mean ± SEM; range, -139 to -47 pA; *n* = 6) and occurred between -10 and 0 mV. The voltage-dependence of steady-state inactivation of the Na^+ current was examined using a two-pulse protocol (Bezanilla and Armstrong, 1977). Cells held at -80 mV were subjected to prepulse potentials ranging from -100 mV to 0 mV for 500 msec, after which a 25-msec test pulse to 0 mV was applied (Fig. A.1.1B). The normalized test current was plotted against prepulse potentials and the resulting curve was fit with a Boltzmann relation (Fig. A.1.B). Half-maximal steady-state inactivation ($E_{1/2}$) of the Na^+ current occurred at -58 ± 2 mV (*n* = 6). Application of TTX, a voltage-dependent Na^+ channel blocker, induced a dose-dependent and reversible reduction in peak Na^+ current amplitude with an estimated ED_{50} of 15 ± 6 nM (*n* = 5; Fig. A.1.2). Concentrations of 100 to 1000 nM TTX completely abolished the inward Na^+ current. These results demonstrate the presence of TTX-sensitive voltage-dependent Na^+ currents in goldfish somatotrophs.

Voltage-dependent Ba^+ currents

In the present study, Ba^{2+} was used as the charge carrier instead of Ca^{2+} for the reasons outlined in Chapter 3. Command potentials more depolarized than -30 mV elicited an inward Ba^{2+} current which was resistant to inactivation during the 200 msec command pulse (Fig. A.1.3). The peak inward current reached 33 ± 7 pA (range = -18.1 to -83.7 pA; *n* = 9) around +10 mV. These data directly demonstrate the presence of high-voltage activated Ca^{2+} currents in identified goldfish somatotrophs.

DISCUSSION

These data demonstrate the presence of voltage-dependent Na^+ currents in identified goldfish somatotrophs. The voltage-dependent activation and inactivation characteristics of the voltage-dependent Na^+ currents in identified goldfish somatotrophs are similar to those observed in identified goldfish gonadotrophs (Chapters 3 and 4). Additionally, Na^+ currents in both identified goldfish gonadotrophs and somatotrophs are sensitive to TTX (Chapter 3 and 4). Although voltage-dependent Na^+ currents have been observed in both goldfish gonadotrophs and somatotrophs, they do not appear to be involved in the regulation of GnRH action. Application of TTX, does not alter basal or GnRH-induced GTH-II or GH secretion (Chapter 4; Van Goor, 1996). The possible involvement of voltage-dependent Na^+ currents in mediating dopamine D1-stimulated GH secretion remains to be elucidated.

In addition to voltage-dependent Na^+ currents, voltage-dependent Ba^{2+} currents were also observed in goldfish somatotrophs. Based on the activation and inactivation properties, the Ba^{2+} currents observed in these cells are similar to L-type currents observed in other pituitary cell types, including goldfish gonadotrophs (Chapter 3 and 5). However, their sensitivity to dihydropyridine Ca^{2+} channel antagonists and agonists remains to be determined. Nevertheless, these results confirm the presence of high-voltage activated, inactivation resistant Ca^{2+} channels in goldfish somatotrophs, first implicated by hormone release studies (Wong *et al.*, 1994a). Preliminary studies indicate that dopamine stimulates an increase in voltage-dependent Ba^{2+} current amplitude (Chapter 5) and $[Ca^{2+}]_i$ in identified goldfish somatotrophs (Van Goor, unpublished observations) . However, whether GnRH stimulates an increase in Ba^{2+} current amplitude or $[Ca^{2+}]_i$ in these cells is not known.

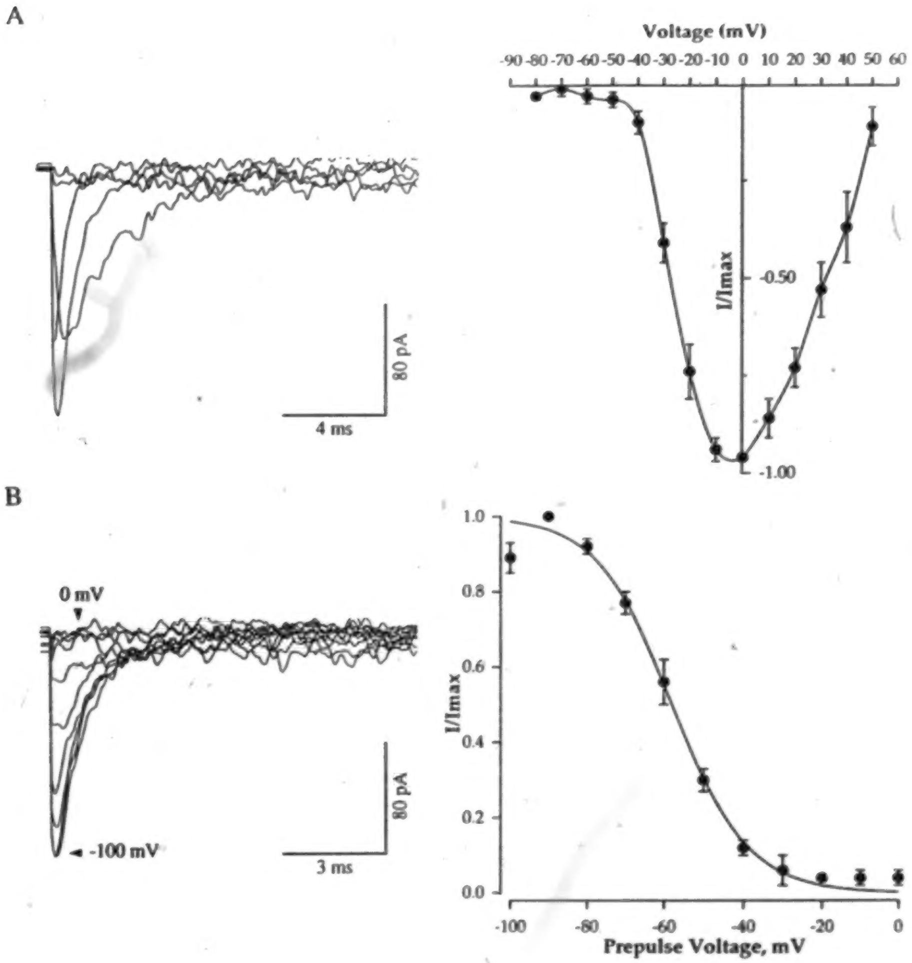

Fig. A.1.1 Transient, voltage-dependent Na$^+$ current in identified goldfish somatotrophs. A. The current-voltage relationship of Na$^+$ current was obtained by holding the cells at -90 mV and then stepping for 50 msec to command potentials between -80 and +50 mV, in 10 mV increments (mean ± SEM, *n* = 6). Shown at left are representative current traces elicited during command potentials to -80 -40, -20, 0 and +20 mV. B. Steady-state inactivation of the voltage-dependent Na$^+$ current (mean ± SEM, *n* = 6). Inactivation curves were generated from experiments where the membrane potential was stepped to between -100 and 0 mV for 500 msec prior to stepping to a test potential of 0 mV for 25 msec. Shown at left are representative current traces of the remaining currents elicited by a test pulse to 0 mV after prepulse potentials between -100 and 0 mV. The solid line for the steady state inactivation curve is a fitted Boltzmann relation (equation 1), where $E_{1/2}$ = -57.9 mV and k = 9.7. All currents were normalized to the maximal inward current.

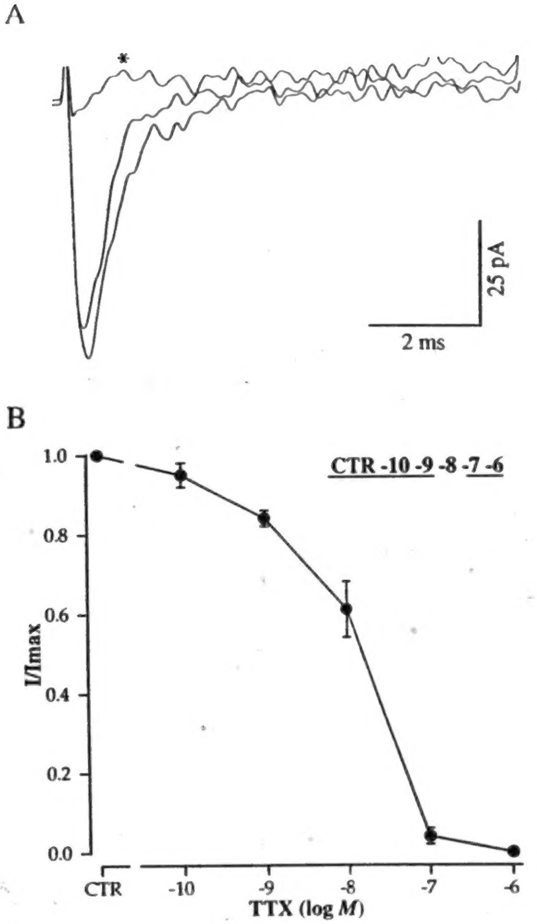

Fig. A. 1.2. TTX inhibits voltage-dependent Na$^+$ currents in identified goldfish somatotrophs. Currents were elicited by a 25 msec voltage step from -90 to -10 mV. A. Current traces from a single somatotroph before, during (*) and after the application of 100 nM TTX. B. Dose-dependent inhibition of Na$^+$ current by 0.01 nM to 1 μM TTX (mean ± SEM; *n* = 5). All currents were normalized to the maximal inward current. Current responses that are not significantly different from one another are identified by the same underscore (ANOVA followed by Fisher's LSD test, *P* > 0.05)

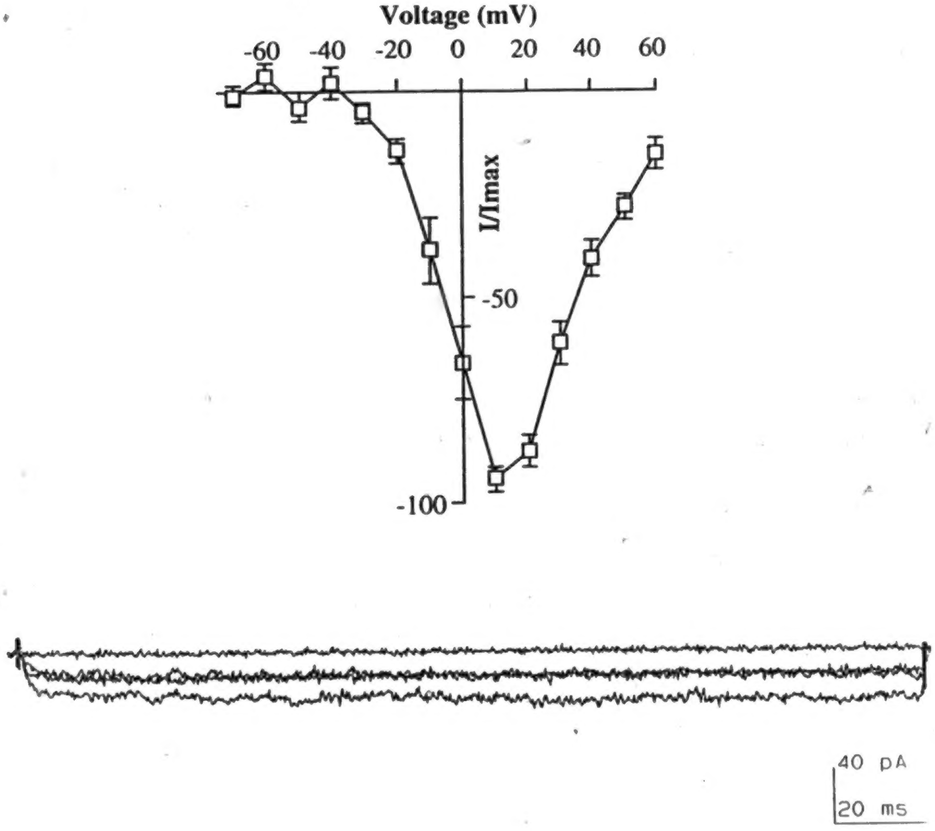

Fig. A. 1.3. Voltage-dependent Ba^{2+} currents in identified goldfish somatotrophs. Ba^{2+} currents were elicited by 200-msec voltage-steps to potentials between -70 and 60 mV from a holding potential of -80 mV. A. Current-voltage relationship of isolated Ba^{2+} currents (mean ± SEM; *n* = 9). All currents were normalized to the maximal inward current. B. Representative Ba^{2+} current traces from a single somatotroph elicited by voltage steps to -70, -10, +10 and +30 mV.

Appendix 2

EFFECTS OF APAMIN ON CGNRH-II-STIMULATED GTH-II SECRETION

INTRODUCTION

In rat gonadotrophs, GnRH receptor activation mobilizes intracellular Ca^{2+} resulting in the generation of oscillatory changes in $[Ca^{2+}]_i$. The resulting transient increases in $[Ca^{2+}]_i$ stimulate apamin-sensitive, Ca^{2+}-activated K channels ($I_{K[Ca^{2+}]}$). This causes a rhythmic hyperpolarization of the plasma membrane, resulting in the removal of Na^+ and Ca^{2+} channel inactivation. In response to a decrease in $[Ca^{2+}]_i$ and the subsequent removal of $I_{K[Ca^{2+}]}$ activation, a slow depolarization of the membrane occurs. This leads to the firing of a few Na^+/Ca^{2+}-dependent action potentials once a threshold potential is reached. However, in identified goldfish gonadotrophs, no $I_{K[Ca^{2+}]}$ was observed in voltage-clamp experiments (Chapter 3). These results suggest that $I_{K[Ca^{2+}]}$ are not involved in GnRH action. To test this hypothesis, the effects of apamin on cGnRH-II-stimulated GTH-II secretion were examined in perifusion experiments.

MATERIALS AND METHODS

Pituitaries from male and female goldfish were excised and their pituitary cells were dispersed and cultured as previously described (Chapter 5). Perifusion studies were performed as described in Chapter 5. Apamin (RBI, Natick, MA) was dissolved in 0.05 M acetic acid. cGnRH-II (Peninsula Lab, La Jolla, CA) was dissolved in double-distilled deionized water.

RESULTS AND DISCUSSION

Application of 1 μM apamin did not alter 100 nM cGnRH-II induced increases in GTH-II secretion (Fig. A.2.1). This preliminary study indicates that cGnRH-II-stimulated GTH-II secretion is not altered by inhibition of $I_{K[Ca^{2+}]}$. It has also been demonstrated that apamin does not affect basal or sGnRH-stimulated GTH-II secretion in goldfish (J. P. Chang, personal communication). These results are consistent with the absence of $I_{K[Ca^{2+}]}$ in goldfish gonadotrophs (Chapter 3).

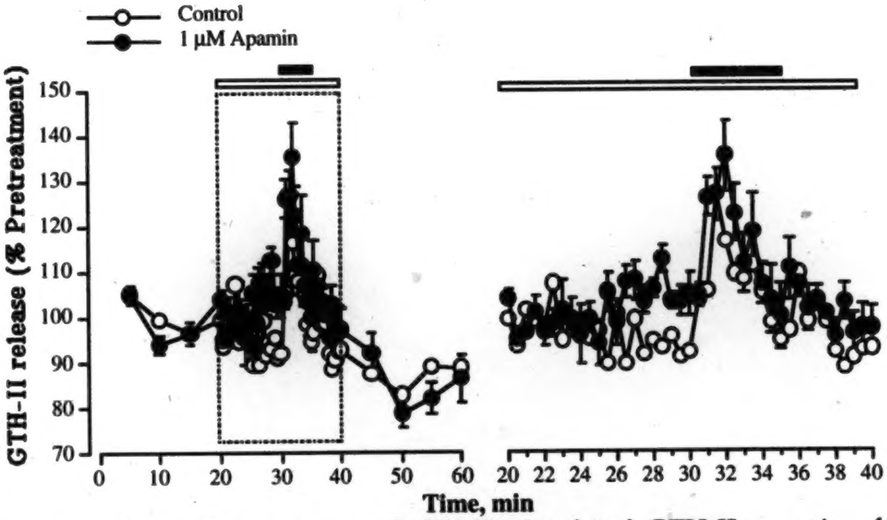

Fig. A.2.1. Effects of apamin on cGnRH-II-stimulated GTH-II secretion from dispersed goldfish pituitary cells in a perifusion experiment. Left panel: GTH-II release response to a 5-min pulse of cGnRH-II (*filled bar*) in the presence (*filled circles*) or absence (*open circles*) of 1 µM apamin (*open bar*). GTH-II release responses (mean ± SEM; n = 2 for control and 4 for apamin) were normalized as a percentage of the pretreatment values (9.31 ± 1.85 ng/ml/2 x 10^6 cells).

Appendix 3

cAMP-INDUCED INCREASE IN VOLTAGE-DEPENDENT CALCIUM CURRENT

INTRODUCTION

Activation of cAMP transduction pathways increase Ca^{2+} influx through voltage-dependent Ca^{2+} channels in cardiac cells, AtT-20 cell line and somatotrophs (Luini *et al.*, 1985; Trautwein and Hescheler, 1990; Chen *et al.*, 1994). In goldfish gonadotrophs, cAMP transduction pathways increases GTH-II secretion from goldfish pituitary cells (Chang *et al.*, 1996). To test the hypothesis that cAMP directly activates voltage-dependent Ca^{2+} channels in goldfish gonadotrophs, the action of the membrane permeant cAMP analog, 8-Bromo-cAMP (8-Br-cAMP), was examined in perforated-patch clamp recordings.

MATERIALS AND METHODS

Male and female goldfish pituitaries were excised and their pituitary cells were dispersed and cultured as previously described (Chapter 5). Gonadotrophs were identified according to their unique cellular morphology (Chapter 2). Nystatin-perforated patch (Korn and Horn, 1989) recording techniques were used as previously described (Chapter 3). The pipette was filled with (in mM) 70 Cs-glutamate, 70 CsCl, 2 $MgCl_2$, 10 HEPES and 160 µg/ml nystatin (pH 7.2). For recordings of Ba^{2+} currents through voltage-dependent Ca^{2+} channels, the external bath solution contained (in mM): 120 N-methyl-D-glucamine-Cl, 20 $BaCl_2$, 1.0 $MgCl_2$, 2.5 KCl, 8 glucose and 10 HEPES (pH adjusted to 7.2 with HCl). The membrane potential for Ba^{2+} currents were not corrected for the liquid-junction potential of 3 mV. 8-Br-cAMP (Research Biochemicals, Natick, MA) was dissolved in saline.

RESULTS AND DISCUSSION

Application of 1 mM 8-Br-cAMP increased the maximal Ba^{2+} current amplitude elicited by a 40-msec voltage step to +10 mV (holding potential = -80 mV) from 125. 0 ± 8.45 pA to 163.8 ± 19.01 pA (Fig. A.3.1A; $P < 0.05$, paired *t*-test, $n = 4$). The current-voltage relationship demonstrated that the stimulatory actions of 8-Br-cAMP occurred between voltage steps from 0 mV to +40 mV; current responses to voltage steps more hyperpolarized that -10 mV were not altered by 8-Br-cAMP (Fig. A.3.1B). These data indicate that cAMP can stimulate an increase in Ca^{2+} entry through voltage-dependent Ca^{2+} channels. This may positively modulate voltage-dependent Ca^{2+} channels to enhance GnRH-stimulated GTH-II secretion. In rat lactotrophs, for example, cAMP has been demonstrated to enhance the plateau phase of TRH-induced increases in $[Ca^{2+}]_i$ (Akerman *et al.*, 1991), suggesting that the activation of two biochemical pathways is important for sustaining $[Ca^{2+}]_i$. Whether a yet unidentified neuroendocrine regulator stimulates cAMP production to positively modulate GnRH-induced increases in $[Ca^{2+}]_i$ or independently increases $[Ca^{2+}]_i$ to stimulated GTH-secretion remains to be determined.

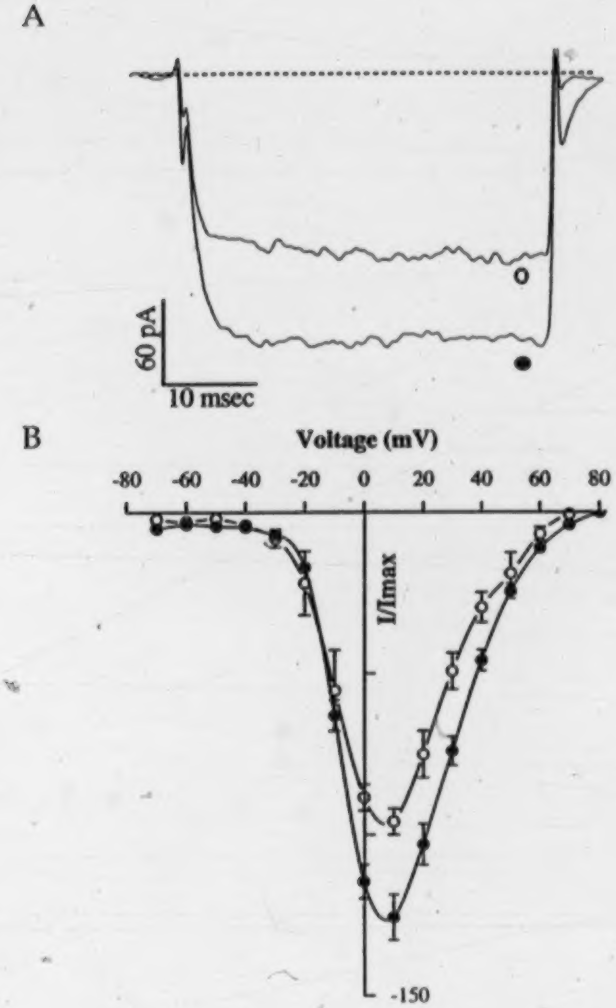

Fig. A.3.1 Effects of 8-Br-cAMP on voltage-dependent Ba^{2+} current in identified goldfish gonadotrophs. A. Superimposed Ba^{2+} currents in the presence or absence of 1 mM 8-Br-cAMP. Currents were elicited by a 40 msec voltage-step to +10 mV from a holding potential of -80 mV. B. Current-voltage relationship of the Ba^{2+} current elicited by 200-msec voltage-steps to the potentials indicated (holding potential = -80 mV) in the presence (*filled circles*) or absence (*open circles*) of 1 mM 8-Br-cAMP. All currents were normalized to the maximal inward current in the absence of 8-bromo-cAMP and are presented as mean ± SEM ($n = 4$).